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Blockade of the Calorigenic, Hyperglycemic, Lactic Acid producing and Fatty Acid mobilizing Effects of Adrenaline by an Adrenergic β-receptor Blocking Agent (Pronethalol) in Experiments on the Rabbit

By

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Abstract

SVEDMYR, N *Blockade of the calorigenic hyperglycemic lactic acid-producing and fatty acid mobilizing effects of adrenaline by an adrenergic β-receptor blocking agent (pronethalol) in experiments on the rabbit* Acta physiol. scand. 1967 71 1—5

Pronethalol in dose of 6 mg/kg greatly inhibited the stimulatory effect of adrenaline given in an intravenous infusion of 0.5 μg/kg min for 60 min, on the oxygen consumption in the rabbit. Pronethalol also blocked the stimulatory action of adrenaline on the lactic acid production, and its hyperglycemic and fatty acid-mobilizing effects. The relationship between these effects are discussed

Adrenergic β-receptor blocking agents have been found, in experiments on both animals and man, to inhibit the stimulatory effects of adrenaline on the mobilization of fatty acids (Pilkington *et al* 1962) and on the production of lactic acid (Lundholm and Svedmyr 1963 1966) and also its cardio-accelerative effects (Powell and Slater 1958 Dornhorst and Robinson 1962) It has also been found that the phosphorylase-activating effect of adrenaline and isoprenaline on striated muscle is blocked by pronethalol (Mohme Lundholm and Svedmyr 1964)

It has been shown that in man the calorigenic effects of adrenaline can be ascribed partly to its lactate and FFA-mobilizing actions (Svedmyr 1966 c) Approximately two-thirds of this effect appeared to be due to an increase in the mobilization and metabolism of lactate and FFA. The calorigenic lactate FFA and glucose-increasing actions of adrenaline was totally blocked in man by the β-adrenergic blocking agent MJ 1999 (Svedmyr and Lundholm 1967)

An intimate relationship between the calorigenic effect of adrenaline and its stimulation of lactic acid production has been demonstrated in experiments

rabbit (Lundholm 1949 Lundholm and Svedmyr 1963 1964 Svedmyr 1966 a b) According to Rudman *et al* (1963) the catecholamines had no FFA-mobilizing effect in experiments on the rabbit in vivo or in vitro Drury (1957) had however found a profound influence of adrenaline on the lipid metabolism of the rabbit. It seemed of interest, therefore to study in the rabbit too how pronethalol influenced the effect of adrenaline on the oxygen consumption and lactate- glucose and FFA-content of plasma.

Method

The experiments were performed on rabbits weighing between 2 and 3 kg The animal had been starved for bout 18 hrs before the xperiment. Under local anesthesia with lidocain fine cannula was introduced into the rtery of one ear and another cannula int the marginal ein of the other ea The animal was then placed i metabolism pparatus which has been described by Svedmyr (1966 d) and in which the O consumption was recorded for 10-min periods The metabolism cage was thermostatically controlled t 21 C. By means of fine tubes which were brought out through the lid of the cage rterial blood could be withdrawn from the arterial cannula and infusions could be given through the cannula in the ural em. When, after about 60 min, the animal had become acclimatized to the metabolism cage nd the O_2 consumption had become stable the basal O_2 consumption wa determined for period of 60 min. Adrenaline in dose of 0.5 µg/kg/min was then inf sed for 60 min i v the adrenaline (base) being dissolved in 20 ml sterile pyrogen-free 0.9 % NaCl solution to which 0 1 % ascorbic acid was added to prevent oxidation In the pronethalol experiments 6 mg pronethalol (Alderline I 1) per kg body weight were first infused for 10 min before the adrenaline infusion.

Arterial blood samples were taken immediately before the adrenaline infusion (basal samples) nd also 15 60 and 150 min after its commencement. The lactic acid in whole blood was determined according to Lundholm, Mohme Lundholm and V mos (1963) the blood glucose according to Bergmeyer and Bernt (196) and FFA in 2 5 ml plasma according to Trout, Estes and Friedberg (1960)

Results

Adrenaline The effects of 0.5 µg adrenaline/kg min on the oxygen consumption, lactic acid content, free fatty acids and glucose are shown in Fig 1 and Table I and II It can be seen that the oxygen consumption and lactic acid and glucose contents reached their maxima at the end of the infusion. The FFA content increased initially but this increase was transient In some experiments more frequent blood samples were taken, and in these cases the FFA content was found to have regained its basal alue after 40 min of infusion.

Pronethalol + ad enaline After pronethalol infusion, the adrenaline continued to increase the glucose lactic acid and FFA contents, but this stimulatory effect was considerably reduced (Fig 1 Table II) The stim latory effect on the oxygen consumption wa almost complet ly blocked (Table I) In the pronethalol-adrenaline experiments the FFA content was ncreased by 41 ± 13 µeq l (P<0 02) over the basal value 60 m n after the start of the adrenaline infusion (Fig 1) This increase was greater than that noted at the same time in the experiments with adrenaline alone the differenc being 35 ± 16 µeq l (P<0.05 Björntorp (1964) found that pronethalol timulated the liberation of fatty acids from adipose tissue and it is therefore pos ibl that thi difference was due to an individual effect of the pronethalol itself

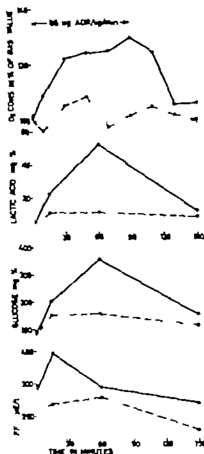


Fig. 1 The influence of pronethalol (6 mg/kg) on the effect of adrenaline on the O_2 consumption, and lactic acid, glucose and free fatty acid contents in the blood. Whole lines refer to adrenaline alone, and broken lines to the effect of adrenaline after pronethalol. Mean of 8 resp. 7 expts

TABLE 1. The effect of adrenaline and pronethalol + adrenaline on the oxygen consumption of rabbits. N = number of tests. P = probability that the effect was due to chance

	Basal values ml kg/60 min	Increase 0–120 min after adrenaline; per cent of basal values
Control N = 8	512 ± 19	+23.5 ± 3.7 P < 0.001
Pronethalol N = 7	498 ± 17	+3.7 ± 1.6

Difference adrenaline − pronethalol + adrenaline = 19.8 ± 4.0
P < 0.001

TABLE II The effect of adrenaline and pronethalol + adrenaline on lactic acid, glucose nd free fatty acid content of the blood in the rabbit Maximal increases of lactic acid nd glucose contents were reached 60 min after the start of the infusion and after 15 min in the case of free fatty cids. P = probability that the effect was due to chance

	Basal value	Maximal increase over basal alue after adrenaline
Control		
Lactic acid mg per cent	4.5 ± 0 8	50.8 ± 6.0 P < 0.001
Glucose mg per cent	82.5 ± 5 6	278 ± 29 4 P < 0.001
Free fatty cids µeq/l	282 ± 29 0	100 ± 22.0 P < 0 001
Pronethalol		
Lactic acid mg per cent	6 7 ± 0.7	4.6 ± 0 7 P < 0.001
Glucose mg per cent	82.2 ± 6 8	76 ± 14.0 P < 0 01
Free fatty acids µeq/l	15 ± 28	22 _ 5.7 P < 0 01

Difference drenaline — pronethalol + adrenaline

lactic acid	46 2 ± 6.0	P < 0.001
glucose	202 ± 33 0	P < 0.001
free f t y acids	78 ± 22 5	P 0.01

D scussion

Pronethalol greatly inhibited th elevating effect of adrenaline on the content of lactic acid, glucose and free fatty acids in the blood. The tim lation of the oxygen onsumption by adrenaline wa almost complet ly blocked by pronethalol. S nce in pre us experiment on the rabbit (Lundholm 1949 S edmyr 1966 a b) a close relation hip ha been found bet een ncreased lactic cid oxidation and stimulation of the oxygen consumption after adrenaline medication, it eems probabl that pronethalol i hibited the l rigenic effect of adrenal n partly by blocking its tim latory a tion on the la tic ac d production. The FFA mol lizing effect of adrenal ne wa also blocked a d th effect may contribute to the reduction of the calorigen effect F om the exp riment t d fficult t judge the r lative importanc of an inc eased lactat respect FFA-met l sm f r the lorigenic effect of adrenal ne i th rabbit

After electr blockade of the FFA-mol lizing effect of drenal ne by nicotinic acid Lundh lm and S edmy (to be publi hed observed that the alorigenic effect was reduced during the first 60 m l r ther w n t ffected The reduct on of the calorigenic ffect w of the order of bout 40 per cent of th t ta l ffect

According to Rudman *et al* (1963) adrenaline had no FFA-mobilizing effect in vivo in the rabbit. They however determined the effect 150 min after the injection of adrenaline when according to my results its effect had disappeared. This transit effect of adrenaline may also explain that Rudman *et al* (1963) in in vitro test on perirenal fat tissue from the rabbit failed to find any FFA-accumulation in the tissue after 3 hrs incubation with adrenaline.

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The In Vitro Catabolism of Histamine by Sheep Liver Tissue

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Abstract

SJAASTAD, O. V. *The in vitro catabolism of histamine by sheep liver tissue.* Acta physiol. scand. 1967. *71*. 6—15.

The *in vitro* inactivation of histamine by the liver of sheep has been examined with the following results. At optimal substrate concentrations liver slices inactivated 178.5 ± 57.4 (S.D.) μg histamine diphosphate/g wet weight of tissue/hr. There was no marked difference between the histamine inactivation [illegible] liver slices and homogenates.—Aminoguanidine, a powerful inhibitor of diamine oxidase (E.C. 1.4.3.6.) strongly counteracted the histamine inactivation, indicating that oxidative deamination is the major pathway for histamine inactivation in sheep liver tissue *in vitro*.—On an average about 1 per cent of added histamine was inactivated by conjugation.—Inhibition experiments indicated that no significant methylation takes place.—The average content of preformed histamine in 41 sheep was 24.7 ± 11.1 μg histamine diphosphate/g wet weight of tissue.

When large amounts of histamine were given to sheep by mouth, the biological activity disappeared rapidly from the rumen (Sjaastad 1967a), but it was not certain to what extent this was due to absorption of free histamine by the rumen epithelium or to inactivation of histamine by rumen content (Sjaastad 1967a). *In vitro* observations indicated that the inactivating capacities of the rumen epithelium and the blood interposed between the rumen and the liver would be exceeded if large amounts of unchanged histamine were absorbed from the rumen (Sjaastad 1967b [illegible]). Since under such conditions considerable amounts of histamine would reach the liver unchanged, examinations of the capacity of sheep liver tissue to inactivate histamine were undertaken. Studies on the nature of this inactivation were also carried out.

Methods and material

Livers of both male and female adult sheep of the D[illegible]la breed were obtained [illegible] slaughterhouse. Pieces of 10—15 g of liver were transferred to thermosflasks containing partly frozen Ringer-phosphate solution (pH 7.4) containing glucose 0.2 per cent. Generally the pieces of liver were transferred to Ringer solution within 10 min. of death.

Preparation of liver tissue

Slices with an average thickness of 0.3—0.4 mm were conventionally prepared from chilled liver

Liver homogenates were obtained by mincing the tissue in a glass homogenizer (Griffith tube, Baird & Tatlock, Ltd., London) containing small amounts of Ringer's solution. The mixture was then *transferred quantitatively to* a vibratory disintegrator (Mickle Laboratory Engineering Co., Gomshall, Surrey) and further disintegrated for 2 min with Celite (60—80 mesh). The homogenates were centrifuged for 3 min at 3,000 × g and the supernatant fluid was diluted with Ringer's solution to the desired final volume.

Determination of the concentration of free and conjugated histamine in liver tissue

Free histamine. Pieces of liver tissue kept in partly frozen Ringer's solution were heated at 80° C for about 2 min as soon as possible after slaughter and always within 1 hr of death. Samples of 1 g were homogenized and made up to 25 ml with Tyrode's solution. The histamine content was determined directly on isolated guinea-pig ileum, suspended in Tyrode's solution containing atropine (0.05 mg/l). Antihistamine was occasionally added to the organ bath to confirm the specificity of the assay. The tissue extracts could be left at +4° C for 24 hrs without loss of histamine activity.

Conjugated histamine. Two slightly different methods were used.

1) Homogenized samples of heated liver tissue (1 g) were diluted to 20 ml with distilled water. The homogenate was treated with two volumes of acetone and centrifuged at 3,000 × g for 5 min. After addition of 2.0 ml of N HCl to the supernatant providing a pH below 2, it was concentrated to 2—3 ml *in vacuo*. The residue was dissolved in 10 ml of 10 N HCl and boiled under reflux for 3 hours. After centrifuging at 3,000 × g for 5 min, the hydrolysate was evaporated to absolute dryness *in vacuo*. The residue was dissolved in 8—9 ml of distilled water, neutralized with N NaOH and after adding distilled water to a final volume of 10 ml, the histamine concentration was determined on guinea-pig ileum. The values obtained represent total histamine (free+conjugated).

2) After homogenization of heated liver tissue, aliquots of 2 g were diluted to 20 ml with distilled water and incubated for 30 min at 37° C in the presence of diamine oxidase, an enzyme which oxidizes free histamine but does not act upon N-acetylhistamine (Kapeller—Adler 1965). The mixtures were then heated at 80° C for 5 min (to destroy the diamine oxidase) and centrifuged for 3 min at 3,000 × g. The supernatant was divided into two equal parts. To check that zero values of free histamine were obtained by incubation with diamine oxidase, one part was adjusted to pH 6.5 and subjected to ion-exchange chromatography using the principles outlined by Dunér and Pernow (1956). The other part was treated with acetone and hydrolyzed as described under 1. After centrifugation the hydrolysate was evaporated to dryness, adjusted to pH 6.5 with N NaOH and subjected to ion-exchange chromatography (*Dunér and Pernow 1956*). By this method the values for conjugated histamine were obtained directly or after subtracting the minute quantities of free histamine which occasionally were left.

Determination of histamine inactivating capacity

Four ml of liver homogenate corresponding to 200 mg wet weight of tissue, or 200 mg of liver slices suspended in 4 ml of Ringer's solution, were transferred to Warburg reaction vessels. After adding 0.1 ml of Ringer's solution containing 160 µg of histamine diphosphate, the Warburg vessels were gassed for 1 min (95 % O_2—5 % CO_2) and the mixture was incubated at 37° C for 2 hrs. Duplicate samples of liver homogenates were heated to 80° C and the histamine concentration was determined to obtain the total amount of histamine initially present in incubated homogenates. Also when liver slices were incubated, the amount of histamine added (160 µg) was corrected for preformed histamine in the tissue (determined as described above).

After incubation for 2 hrs, histamine inactivation was terminated by rapid heating of the incubation mixture to 80° C in a water bath, and the remaining histamine was determined. The values for histamine inactivating capacity are expressed as µg of histamine diphosphate inactivated/g wet weight of liver/hr at 37° C. The coefficient of variation for the method determined from the difference between duplicates was 7.3 per cent.

Experiments with inhibitors

The inactivation of histamine was slow when liver tissue and added histamine were incubated in the presence of aminoguanidine or iproniazid. Since the calculation of the rate of inactivation was based on the determination of differences in histamine concentration, the ratio *tissue incubated/histamine added* was increased in experiments with inhibitors compared to that used in experiments without inhibitor. The changes in histamine concentration were then sufficiently large to be accurately determined. Therefore experiments in the presence of aminoguanidine or iproniazid were carried out as follows. To Warburg vessels containing 3 ml of liver homogenate (corresponding to 1 g of liver tissue) or 1 g of liver slices suspended in 3 ml of Ringer's solution, 1 ml of Ringer's solution containing aminoguanidine 3 × 10^{-5}—3 × 10^{-3} M was added and the mixtures left for

equilibration for 10 min. Incubation and determination of histamine inactivation was carried out as described bove af er adding 0.1 ml of Ringer' solution containing 200 μg of histamine diphosphate/ml.

Inhibitors of diamine oxidase (histaminase) re know to interfere with the biological determination of histamine on guinea-pig ileum by potentiation (Mongar and Schild 1951 Arunlakshana, Mongar and Schild 1954) Therefore in some experiments where aminoguanidine had been added t the incubation medium the histamine left after incubation was also determined after ddition of such concentrations of minoguanidine to the Tyrode solution that the amounts of inhibitor present in the test-samples became negligible i comparison. N difference between such parallel assays was detected.

Formation of conjugated histamine by liver tissue

Pilot experiments showed that small amounts of conjugated histamine were formed when dded histamine was inactiv ted by sheep liver tissue T obtain the formation of quantities sufficiently large to be easily determined it was therefore necessary t incubat liver tissue with large mounts of histamine Relatively short incubation periods were used t avoid bacterial growth since some bacteria probably have the bility to conjugate histamine (Urbach 1949) Large amounts of liver tissue (3—5 g) were incubated so that the histamine added (1,000—1,500 μg) was inactivated within 1 1/2—3 hrs. As further precautions to void growth of bacteria, merthiolat (10- g/ml) was added to the incubation medium nd all equipment used was sterilized. I few experiments bacteriological examination was carried out after the incubation. The liver tissue was incubated until free histamine could no longer be detected in the incubation fluid by direct bioassay The incubation mixture was then heated t 80° C, diluted to 20 ml with distilled water homogenized, nd divided i two equal parts. Free nd conjugated histamine was determined as described for conjugated histamin in tissue (method 2)

All histamine values in this paper are uncorrected and refer to histamine diphosphat (M.W 307 compared with 111 for the base) In most cases they represent th mean of duplicate experiments. The histamine assays were carried out with coded samples. The variability of histamine values is given as standard deviation (S.D)

Ion-exchange resin

Ion-exchange was carried out with Amberlit IRC-50 (Standard Grade) from British Drug Houses, Ltd, England. Batches of the resin were prepared according to Bergstrom nd Hansson (1951)

Substances

Histamine diphosphate and N-acetylhistamine were obtained f om Nutritional Biochemicals Corp., Cleveland, Ohio. An antihistamine Allergin® (Diphenhydramine chloride) was purchased from Nyegaard & Co., A/S, Oslo. Aminoguanidine bicarbonat from L Ligh & Co. Ltd., Col brook was used Iproniazid (1-isopropyl-2-isonicotinylhydrazine) was btained from Jura, Milano. Diamine oxidase (from hog kidney) S-adenosylmethionine cetyl-CoA and p-Hydroxymercuribenzoa (PHMB) from Sigma Chemical Co St. Louis, were used.

Results

Concentration of free and conj gated histamine in liver tissue

Free histamine

In 41 heep the a erage concentration of free histamine was 24 7 ± 11 1 μg/g wet weight of li er tissue Low ncentrations of histamine were found if the tis ue was homogenized prior to heat ng

Conjugated histamine

In 6 animal examined the concentration of total histamine wa of the same order as free histamine. This indicates that conjugated histamine is not normally present in detectable concentrations in sheep liver

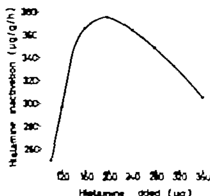


Fig. 1 Inactivation of histamine in liver homogenate at varying substrate concentrations. Each value represent the mean of duplicate incubations. Incubation period 2 hrs.

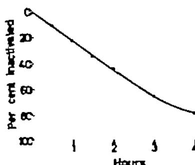


Fig. 2. Inactivation of histamine in liver homogenate. Amount of tissue 200 mg. Initial amounts of histamine 160 µg. The curve is based on mean values from 4 experiments.

The histamine inactivating capacity of liver tissue

Preliminary experiments showed that when 200 mg of homogenized liver was incubated with histamine for 2 hrs, maximum inactivation was obtained when the amounts of added substrate were in the range 160—240 µg. The results of one out of three experiments are given in Fig 1. When 160 µg of substrate was added the rate of histamine inactivation was fairly constant during incubation periods of 2 hours (Fig 2). The figures given for histamine inactivation in this paper are therefore regarded as being almost identical with the *in vitro* inactivating capacity of the tissue.

In 42 animals the average inactivating capacity of liver slices was 178.5 ± 57.4 µg/g wet weight/hr at 37 C. No marked difference was found between the inactivating capacities of male and female sheep. In 8 sheep the average histamine inactivating capacity of liver homogenates was 146.4 ± 39.5 µg/g/hr compared with 172.1 ± 35.5 µg/g/hr in liver slices from the same animals (Table I). This difference is not statistically significant ($p > 0.05$).

TABLE I Comparison between histamine inactivation in liver slices and liver homogenates. Effect of aminoguanidine on the inactivation of histamine in liver tissue. Incubation period 2 hrs. In experiments without aminoguanidine the initial amounts of added histamine were 160 µg and in experiments with aminoguanidine 20 µg

Inactivation of histamine (µg/g/hr)

Liver slices		Liver homogenates	
Without aminoguanidine n = 8	Aminoguanidine (10^{-4}M) n = 20	Without aminoguanidine n = 8	Aminoguanidine (10^{-4}M) n = 19
172.1 ± 33.5	5.0 ± 1.8	146.4 ± 39.5	3.6 ± 1.1

Effect of inhibitors on the inactivation of histamine in liver tissue

Aminoguanidine is known to be a potent inhibitor of diamine oxidase (histaminase) (Schuler 1952). When sheep liver tissue was incubated in the presence of aminoguanidine the inactivation of histamine was slow (Table I). Liver homogenates inactivated histamine at a significantly slower rate in the presence of aminoguanidine than liver slices ($p < 0.01$). The rate of histamine inactivation did not change detectably when the concentration of aminoguanidine was varied between 10^{-4}M and 10^{-3}M. When liver slices were incubated without inhibitor with the same amount of histamine (20 µg) as used in the experiments with aminoguanidine, no histamine was detected after incubation for 2 hrs.

In some experiments the disappearance of histamine in aminoguanidine treated liver homogenates (20 µg histamine added) was compared with that in boiled control (16 expts). In order to reduce the effect of individual variation in the rate of histamine inactivation liver tissue from only three sheep was used for these experiments. The average quantities of histamine left after incubation of aminoguanidine treated and boiled samples were 14.4 ± 2.2 µg and 19.8 ± 1.3 µg respectively. This difference is statistically significant ($p < 0.001$). The amounts of histamine left in boiled homogenates after incubation were also significantly lower ($p < 0.05$) than the initial quantities 21.3 ± 0.9 µg.

Iproniazid inhibits both monoamine oxidase (Zeller, Barsky and Berman 1955) and diamine oxidase (Lindell *et al.* 1960). When both aminoguanidine and iproniazid were added to liver tissue the histamine inactivation did not deviate markedly from that in samples to which only aminoguanidine was added (Table II).

PHMB is reported to be a powerful inhibitor of the methylation of histamine (Lindahl 1960). Addition of this substance to incubates of liver tissue did not change the histamine inactivation detectably. A small inhibition by PHMB would, however

TABLE II. Effect of iproniazid on histamine inactivation by aminoguanidine-treated liver tissue
Initial amounts of histamine 20 μg Incubation period 2 hrs

Expt. no.	Method for preparation of tissue	Inactivation of histamine (μg/g/hr) Aminoguanidine (10^{-4}M)	Aminoguanidine (10^{-4}M) Iproniazid (10^{-3}M)
1	slices	6.0	4.4
2	slices	8.3	8.0
3	slices	6.5	6.5
4	homogenate	4 6	4.6
		Mean 6.4±1.5	5.9±1 7

be more easily detected if the histamine inactivation was reduced by aminoguanidine. Therefore, in 5 expts. PHMB was added to aminoguanidine-treated samples of liver slices. The mean inactivation of histamine was 4 4±0 4 μg/g/hr This is not significantly different (p>0 05) from the inactivation in liver slices to which only aminoguanidine had been added (5 0±1.8 μg/g/hr)

S-adenosylmethionine is reported to be essential for methylation of histamine in liver homogenates (Lindahl 1958 a) Addition of this substance to aminoguanidine treated liver homogenates (3 expts.) or liver slices (2 expts,) did not markedly change the rate of histamine inactivation.

Conjugation of histamine by liver tissue

The effect of merthiolate on the inactivation of histamine in liver tissue during short incubation periods (2 hrs) was examined in preliminary experiments. When liver slices were incubated with histamine (160 μg) and merthiolate (4 expts.) the histamine inactivating capacity was not markedly different from samples to which merthiolate had not been added (151 and 159 μg/g/hr respectively) The inactivation of histamine by liver slices to which both aminoguanidine and merthiolate had been added (17 expts.) was 4 4±2.3 μg/g/hr compared with 5 9±2.7 μg/g/hr in aminoguanidine-treated samples of the same animals (15 expts.) This difference is not significant (p>0 05)

In the 20 animals examined there was demonstrable formation of conjugated histamine by liver tissue. On an average 0 9±0 5 per cent of the histamine added had been conjugated. There was no marked difference in the amounts conjugated by liver homogenates (3 expts) and liver slices. The addition of acetyl-CoA did not result in increased formation of conjugated histamine (Table III)

In some experiments merthiolate was replaced by chloramphenicol The amounts of conjugated histamine found in these experiments were of a similar order as those found when merthiolate was used. In the experiments in which bacteriological examinations were made, the incubation mixture was found to be sterile at the end of the incubation period.

TABLE III Effect of acetyl-CoA on the formation of conjugated histamine by liver tissue. Histamine added: 1,000 μg. Amount of tissue: 5 g

Expt. no.	Preparation of tissue	Conjugated histamine formed (calculated as μg histamine diphosphate)	
		Without acetyl—CoA	Acetyl—CoA
1	Homogenate	8.5	8.2
2	Homogenate	8.6	9.9
3	Slices	13.4	14.4

TABLE IV Stability of acetyl histamine in liver slices. Period of incubation: 2 hrs. Amount of liver tissue: 1 g. The values for N-acetylhistamine are given as histamine diphosphate

Expt. no.	Preparation of tissue	Added N-acetyl-histamine (μg)	Free histamine present in tissue before incubation (μg)	Free histamine present after incubation (μg)	Conjugated histamine after incubation (μg)	Per cent of added N acetylhistamine disappeared
1	homogenat	80	12.9	2.4	69.9	12.6
1	slices	80	16.8	10.5	75.4	5.6
2	homogena	80	11.5	1.2	73.0	8.8
2	slices	80	19.5	11.3	74.3	7.1

Acetylated arylamines may be deacetylated in the liver of several species (Leibmann and Anaclerio 1961). Krebs, Sykes and Bartley (1947) have suggested that the formation of acetylarylamine is a result of a balance between the activities of acetylating and deacetylating systems. It has further been shown that acetylhistamine is deacetylated by human faeces (Sjaastad 1966). The possibility existed that conjugated histamine (which is believed to be identical with acetylhistamine) might also be deconjugated in liver tissue when free histamine reached low concentration. As the histamine then formed might be inactivated by other enzymes than the acetylating one, the amount of conjugated histamine found would not give reliable information on the importance of the acetylating process. Some experiments were therefore carried out to examine the stability of acetylhistamine when incubated with liver tissue. In none of the experiments was the decrease in acetylhistamine sufficiently large to be regarded as significant (Table IV).

Discussion

The capacity of liver tissue to inactivate histamine varies widely between species. The liver is the principal site for histamine inactivation in the mouse (Schayer 1953), whereas liver tissue of dogs can inactivate little, if any, histamine (Best and

McHenry 1930) The present study has shown that sheep liver tissue will rapidly inactivate added histamine *in vitro* From the present *in vitro* results a sheep liver weighing 1 kg should be able to inactivate on an average about 180 mg histamine per hour This clearly represents a large reserve capacity for the detoxication of histamine entering the portal system.

Several authors have demonstrated a lag time of 3—5 hrs before any histamine was inactivated in liver tissue *in vitro* (Best and McHenry 1930, McHenry and Gavin 1932 Cotzias and Dole 1952) Possibly the histamine inactivation by sheep liver tissue could increase if exposed to the substrate for a longer time than used by us. However the rate of histamine inactivation in the present experiments was high and almost linearly related to time during incubation for 2 hrs. It is therefore unlikely that the rate of histamine inactivation would have increased much by prolonged incubation. The discrepancy between the results obtained in this study and those reported by the afore mentioned authors might be due to differences in methods. Although somewhat remote, the possibility also exists that the liver of ruminants is exposed to histamine to a larger extent *in vivo* than the liver of single-stomached animals.

Low concentrations of aminoguanidine completely abolish the inactivation of histamine by diamine oxidase (histaminase) (Schuler 1952, Waton 1956) Methyltransferase, the other major histamine metabolizing enzyme (Schayer Kennedy and Smiley 1953 Schayer and Cooper 1956 Schayer and Karjala 1956) is not affected by minoguanidine (Lindahl 1960) and only concentrations of 10^{-3}M or higher influence the formation of histamine in animal tissue (Waton 1956) Since the larger part of the inactivation of histamine in the present experiments was abolished by aminoguanidine in low concentrations, it is likely that oxidative deamination is the principal pathway for the *in vitro* inactivation of histamine in the liver of sheep

But even in the presence of aminoguanidine, sheep liver tissue inactivated histamine, the inactivation being on an average about 3 per cent of that without inhibitor (T ble I) Since low concentrations of histamine were used in the inhibitor experiments t is possible, however that the capacity to inactivat histamine in the presence of aminoguanidine is larger than the results indicate.

It has been reported that histamine is inactivated by methylation in slices of mouse liver but not by mouse liver homogenates unless S-adenosylmethionine is added (Lindahl 1958 a, b) In the present tudy no significant difference between the rates of histamine inacti ation in liver slices and homogenates was found (Table I) and further ddition of S-adenosylmethionine did not change the rate of inactivation detectably These results and the failure of the nhibitor of methyl transferase PHMB, to affect histamine inacti ation when added to aminoguanidine treated l er slices, uggest that little, if ny methylation of histamine takes place *in vitro* n the liver of sheep Of the species previously examined only the liver of rat was nable to methylat histamine (Lindahl 1960)

I *ti* conjugatio of histam ne has been demonstrated in liver homogenates of pigeons and rabbits (Millican, Rosenthal and Tabor 1949) and homogenates of

TABLE III Effect of acetyl-CoA on the formation of conjugated histamine by liver tissue. Histamine added 1 000 µg. Amount of tissue: 5 g

Expt. no.	Preparation of tissue	Conjugated histamine formed (calculated as µg histamine diphosphate)	
		Without acetyl—CoA	Acetyl—CoA
1	Homogenat	8.5	8.2
2	Homogenate	8.6	8.9
3	Slices	15 4	14 4

TABLE IV Stability of acetyl histamin in liver slices. Period of incubation 2 hrs. Amount of liver tissue 1 g. The values for N-acetylhistamine are given as histamine diphosphate

Expt. no.	Preparation of tissue	Added N-acetyl-histamine (µg)	Free histamine present in tissue before ncubation (µg)	Free histamine present after incubation (µg)	Conjugated histamine af er incubation (µg)	Per cent of added N acetylhistamine disappeared
1	homogenate	80	12 9	2.4	69 9	12.6
1	slices	80	16 8	10.5	75.4	5.6
2	homogena	80	11.5	1.2	73.0	8.8
2	slices	80	19 5	11 5	74 3	7 1

Acetylated arylamines may be deacetylated in the liver of several pecies Leibmann and Anaclerio 1961) Krebs, Sykes and Bartley (1947) have suggested that the formation of acetylarylamine is a result of a balance between the activities of acetylating and deacetylating systems. It has further been shown that acetylhistamine i deacetylated by human faeces (Sjaastad 1966) The possibil ty existed that conjugated histamine which is believed to be identical with acetylhistamine) might also be deconjugated in liver tissue when free histamine reached low concentration As the histamine then formed might be inacti ated by other enzymes than the acetylating one the amount of conjugated histamine found would not give reliable information on the importance of th acetylating process. Some experiment were therefore carried out to examine the tability of acet lhistam ne when incubated with liver tissue In non of the experiment wa th decrease in a etyllhistamine ufficiently large to be regarded significant Tabl IV

Discussion

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From the Institute of Physiology University of Oslo, Norway

Pressure Relationships in the Bone Marrow Vascular Bed

By

KÅRE MICHELSEN

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Abstract

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The bone marrow tissue pressure was measured indirectly in anesthetized rabbits by measurement of th intramedullary enous pressure through vein from the bone marrow of the tibia. It varied between 10 nd 80 cm of saline nd pulsated in synchrony with the arterial blood pressure. The socalled bone marrow pressure was measured through needle inserted blindly int the bone marrow It was equal to the venous pressure, but the procedures performed for measurement of the bone marrow pressure, often led to an increase i the venous pressure Alterations in the arterial pressure and the venous pressure outside the bone caused changes in the same direction i the bone marrow tissue pressure Noradrenaline caused drop, acetylcholin rise in this pressure The bone marrow tissue was found ble t maintain considerable pressure difference in the long axis of the bone It seems tha the bone marrow ca ity as for the pressure relationships, can be onsidered as part of the circula ory ystem, coupled i between the arterioles within the bone marrow nd the veins draining the bone

Knowledge about the dynamics of blood circulation n the bone marrow is scarce although the vascular anatomy of this tissue is well described This lack of informa tion is partly due to the naccessibility of the tissue. Circulation studies are also made difficult by the fact that several arteries supply the marrow in the long bones Brookes and Harrison 1957 Göthman 1960) Similarly the bone marrow vascular bed is dra ned by several veins (Brookes and Harrison 1957 de Marneffe 1951) The arteries and th eins to and from the bones run n the surrounding muscle tissue and it not pos ibl to control flow in all of them, not even after extensive surgical proced res

Certa n anatomi al a pect of the bone marrow tissue and it ascular bed ndicate however that the circulation must d ffer in important ways f om that of most other tis ues The bo n marrow tissue is exceptionally soft and it is rigidly enclosed. The arteries are mor thin-walled than i most other organs, and Yoffey (1965 and Hashimoto 1936 has demonstrated that somewhere in the intramedullary course of these arteries th tunica media hanges abruptly and becomes replaced by a single layer of flattened cell Th enous sy tem con ts of a central venous

channel of great volume capacity This runs from one end of the bone marrow cavity to the other and by means of radiating venules (Ecoiffier *et al.* 1957) it drains the sinusoids and capillaries. A nutrient vein and several veins of different calibre from other regions connect the central venous channel with the outside venous system. On phlebograms the bone marrow veins look highly irregular and tortuous (Gilfillan, Petrakis and Steinbach 1957) Histologically they are built as endothelial tubes, with little connective tissue and no smooth muscle cells in their walls (de Marneffe 1951)

Determinations of blood flow through bone marrow tissue have been attempted by a method of venous effluent collection (Cumming 1962) and by perfusion studies through the nutrient artery (Held and Thron 1962 b) Due to the vascular anatomy of bones such measurements are, however difficult to carry out. Interesting observations on the bone marrow circulation in the rabbit fibula have been made by Brånemark (1959) using a technique with intravital microscopy He found that the blood had a low linear velocity in the sinusoids, and locally the blood stream was sometimes seen to stop completely

Of the different parameters used in the study of bone marrow circulation the socalled bone marrow pressure has gathered most interest. This is the pressure measured through a saline-filled cannula inserted blindly into the bone marrow through a hole in the cortical bone. Cuthbertson, Gilfillan and Bachman (1964) and Stein, Morgan and Reynolds (1957) studied the bone marrow pressure in dogs and stated that it varied greatly from a few mm of Hg to close to the arterial pressure, and that t pulsated in synchrony with the arterial pulsations. Shaw (1964) compared the bone marrow pressure to th intramedullary blood flow which he evaluated with the use of a thermistor probe, and found that these two parameters varied in parallel. In spite of these and many ther investigations uncertainty remains about the nature of the bone marrow pressure. Its relationship to the normal intramedullary tissue pressure and to the pressure in various types of intramedullary vessels is thus not known.

In this work the intramedullary venous pressure has been measured directly The measurements and observations of this parameter have made t possible to evaluate the normal intramedullary tissue pressure. The bone marrow pressure has also been recorded, and ts relationship to the intramedullary venous pressure and to the normal intramedullary tissue pressure has been discussed. Furthermore some aspects of th vascular resistances n this peculiar vascular bed have been examined. Based on the experimental results obtained the dynamics of bone marrow circulation have been discussed.

Methods

General procedures. The experiments were carried out on the left tibia of the rabbi The rabbit tibia has normally macroscopically red marrow in the proximal half and yellow marrow in the distal half

Seventy adult rabbits weighing from 3 0 to 4 5 kg were used. They were anesthetized by i. injection of pentobarbitone (Nembutal, Abbott) in an initial dose of 30 to 50 mg per kg and given an injection of heparin (500 u./kg body weigh) The maintenance dose of pentobarbitone

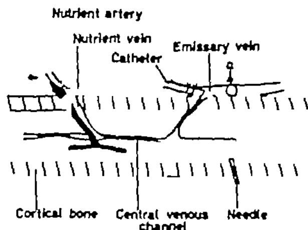


Fig 1 Diagram illustrating th method for measurement of the venous pressures. The pressure in the emissary vein during free flow in this vei ould be measured via the catheter inserted int a side-branch. After occlusion of the emissary ve t the intramedullary venous pressure was measured through the same catheter

was 20 to 50 mg per kg per h The tracheotomized, spontaneously breathing or artificially entilated animal were lying on their right side The left hindlimb wa loosely fastened with semi flexion *i the hip and knee joints.*

The ystemic arterial pressure wa measured through catheter in the left ca otid artery This and other pressures were recorded with pressure transducers (St tham P 23 Dc) on m ltichannel recorder (Electro-Medical Engineering Burbank Calif)

Measurements of the intramedullary enous pressure and of the enous pre sure outside the bone On the lateral border of the rabbit tibia just distal to the tibiofibular synostosis mall vein from the intramedullary enous ystem here called the emissary vein, pierces the cortical bone nd joins the veins of the urrounding soft tissue (Fig 1) A incision was made through the shaved skin on the terolateral side of the leg The tendon of the anterior tibial muscl was t distally The dist l two-thirds of the muscle were then dissected out from the fascia nd turned later lly whereby the nterol teral part of the tibial diaphysis with the extra-osseous part of the emissary ein, was exposed. The emissary ein, which wa not always present n these nimals, ied d meter from 0 4 t 0.8 mm This ein with its ram fications, w dissected out nder dissec ing microscope and 10 mm long polyethylene t bing with a ou er diameter of 0 t mm w inserted into one of is sidebranches. Through thi t bing the collateral pressure in the emissary ei during free flow could be measured By oc lusion of the emissary vein t l F g. 1 the tramedullary enou pressure ould be recorded.

Measurement of the bone marrow pre sure The bone marrow pressure was me red through somewhat thinner needles th usual, but otherwise in the same way as described by others The procedure wa follows bout 0 5 0 5 cm of th urf ce of the ortical bone was exposed nd by means of hand drill hole wa made through the ompact bone A saline filled needle (0 4 mm d 0.t mm d tightly fi t ng the drill hole wa inserted blindly nt the bone marrow After ordinary insertion, the p of h needle stood less tha 1 mm he bone marrow For measurements of bone marrow pressu he enter and in he periphery of the bone marrow ty the needle was inserted arious depth Saline ould be injected nt the bone marrow through the needle When comparisons between he ntramedullary enous pressu and the bone marrow pressure he same region were w ed he needle w inserted into the diaphysis less han l cm from he emissary ei

I order tudy the bon marrow pressure in d fferen egions long he leng xis of he bone marrow y needles were nserted in the diaphysis nd int he prox mal nd distal metaphyses It w l ay hecked pos mortally hat he arious needle tips ere positioned inside the cont nuous smooth walled bone marrow ty

I f sions of noradrenaline and acetylcholine Noradrenaline Nor-eradr & Astra nd acetylcholine hloride (Acetylcholine Roche were nfused int the lef femoral rtery hrough tubing inserted in one f its uscular sidebranches I usion jections ere performed hrough thin catheter in ein Both drugs were used in oncent ion of 100 µg per ml nd infusions t various es were arried out wi h nfusion pump

Method for changing the arterial and enou pressure out de the tibia The femoral rtery nd ei were dissected free for a length of 2 m he middle of he thigh, nd ourniquet pplied around the th gh underneath these essel Arterial nd enou pressures d t t the tourniquet were measured through theters 0 6 mm d in mall rtery nd ein on he surf f or hia to the t bial bone Vari ion he rterial nd venous pres ures distal t he tourniquet were obt ned by the use f screw clamps on he femoral rtery nd the tourniquet region.

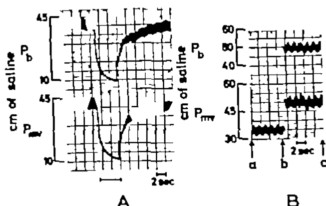


Fig. 2. *A*. Comparison between the intramedullary venous pressure (P_{mv}) and the bone marrow pressure (P_b) in the same region of the tibial bone marrow cavity. During the femoral artery t the limb was occluded. *B*. The effect on the intramedullary enous pressure of the procedures performed for measurement of the bone marrow pressure. Between and *b* the intramedullary venous pressure (P_{mv}) alone was recorded. At *b* saline-filled needle connected to pressure transducer was inserted within one cm from the emissary ein. Further registration (*b* to) showed that the intramedullary venous pressure, which was equal to the bone marrow pressure had increased.

Results

Direct comparison between the bone marrow pressure and the intramedullary venous pressure in the same region. In 20 experiments the bone marrow pressure (P_b) and the intra medullary venous pressure (P_{mv}) in the same region of the tibial bone marrow were compared. The two pressures were found to be nearly equal independent of whether the tip of the bone marrow needle stood in the center or in the periphery of the bone marrow cavity They followed each other also during extreme variations as e.g those produced by occlusion of the arterial supply to the limb (Fig 2 A)

The procedures carried out for measurement of the bone marrow pressure will injure the marrow tissue and it was therefore necessary to find out if these procedures changed the intramedullary ven us pressure. Fig 2 B, which is from a typical experi ment of this type shows that such an effect could occur The intramedullary venous pressure alone was recorded from *a* to *b* At *b* the registration was stopped for one minute, and a saline filled needle connected to a pressure transducer was inserted int the bone marrow On further registration (*b* to) the intramedullary enous pressure, which equalled the bone marrow pressure, was considerably higher than at the outset This effect was seen in most of these experiments, but in som cases the intramedullary venous pressure remained unchanged.

The intramedullary venous pressure Since the procedures performed for measurement of the bone marrow pressure often caused a change in the intramedullary venous pressure this last pressure was studied separately in 20 animals. It varied greatly from one experiment to another and also in one and the same animal in the course of an experiment. Values from 10 to 80 cm of saline were recorded, but usually this pressure was found to be between 25 and 50 cm of saline (the mean systemic arterial pressure being between 100 and 160 cm of saline in these experiments) Th intramed llary enous pressure pulsated in synchrony with the arterial pulsa tions. The pulse pressure aried between 0 and 20 cm of saline. A high pulse pressure was often but not always seen when the ntramedullary venous pressure itself was high.

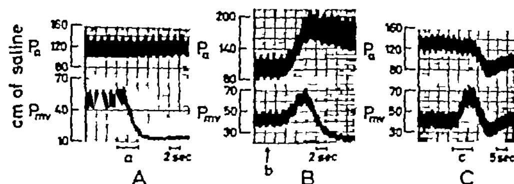


Fig. 3. The effect of noradrenaline and acetylcholine on the intramedullary venous pressure in the tibial diaphysis. *A* During infusion of noradrenaline 10 µg/min, into the femoral artery. *B*. At *b* injection of 30 µg noradrenaline int an ear vein. *C*. During infusion of 20 µg acetylcholine/min into the femoral artery.

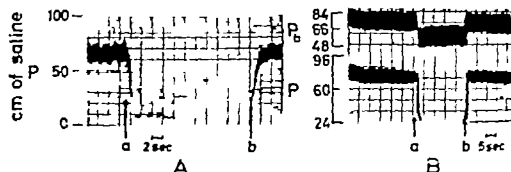


Fig. 4. *A* Comparison between the intramedullary venous pressure and the venous pressure in emissary vein outside the bone. Initially the emissary vein was occluded (see Fig. 1) and the intramedullary venous pressure recorded. At *a* the emissary vein was opened and the pressure in this vein during free flow measured. At *b* the emissary vein was reoccluded. *B*. A similar experiment performed with measurement also of the bone marrow pressure (P_b) in the same region.

There was no systematic relationship between the height of the systemic arterial pressure and the height of the intramedullary venous pressure from one experiment to another. A change in the systemic arterial pressure ... an experiment was usually however mirrored by a change in the same d... the ...dullary venous pressure.

The effect of noradrenaline and acetylcholine on th... ...of noradrenaline 10—30 µg/min into the fe... medullary venous pressure with disappearan... tion of 30 µg of noradrenaline into an ear vei... arterial pressure and a simultaneous rise i... The latter then declined with a decrease in th... sure stayed high.

Infusion of acetylcholine chloride (5—20 µ... initial rise in the intramedullary venous pres... pressure started to fall and the intramedulla...

The intramedullary venous pressure and the pressure in the veins outside the bone Fig 4 A shows a recording of the intramedullary venous pressure. At *a* the emissary vein was opened and the collateral pressure during a period of free flow was measured. At *b* the emissary vein was reoccluded. This and similar measurements revealed that a difference was present between the pressure in the intramedullary veins and the pressure in the emissary vein outside the bone. The pressure in the emissary vein outside the bone varied between 5 and 25 cm of saline with pulse pressure values between 0 and 5 cm of saline. Through the dissection microscope the blood flow in the emissary vein was observed to be directed out of the bone and usually with visible pulsations. Venous valves were often present in the emissary vein.

It was necessary to find out whether or not the occlusion of the emissary vein for measurement of the intramedullary venous pressure caused a venous stasis, leading to an artificially great difference between the intramedullary and extramedullary venous pressure. Five preparations, in which the procedures for bone marrow pressure registration left the intramedullary venous pressure unchanged, were selected. The two pressures were recorded simultaneously from the same marrow region and found to be about equal (Fig 4 B) At *a* the emissary vein was opened and the lateral pressure in it recorded during the period of free flow This opening of the vein caused a fall in the bone marrow pressure, which must still be presumed to equal the intramedullary venous pressure. The bone marrow pressure was, however still higher than the lateral pressure in the emissary vein and its pulse pressure remained high. At *b* the emissary vein was reoccluded whereby the bone marrow pressure again increased. The results from the other four experiments were similar It was therefore concluded that although the occlusion of the emissary vein did bring about an element of regional venous stasis, the intramedullary venous pressure was higher and much more pulsating than the pressure in the veins outside the bone

The influence on bone marrow pressure of the venous pressure and of the arterial pressure outside the bone The influence on bone marrow pressure of changes in the venous and arterial pressure outside the bone was studied in 20 preparations. The pressure in the veins outside the bone, as measured in a small vein, was increased by total occlusion of the femoral vein by the tourniquet technique described (see Methods) The effect is demonstrated in Fig 5. The bone marrow pressure and the pressure in the small artery close to the bone started to rise at the same time as the pressure in the small vein. The venous pressure increased from a low initial value but with a great slope. The bone marrow pressure rose from a higher initial value but with a lesser slope. It will be noted that the bone marrow pulse pressure decreased in the course of the stasis. On removal of the stasis the bone marrow pressure dropped below the pre-stasis level to which it then returned within 40 sec

Plotting of the values for the rising bone marrow and arterial pressures against the simultaneous pressure values from the vein outside the bone gave curves convex towards the venous pressure axis. In Fig 6 A pressure values from an experiment of the type illustrated in Fig 5 have been plotted in this way The bone marrow pressure became equal to the venous pressure at *d* when a considerable difference was still

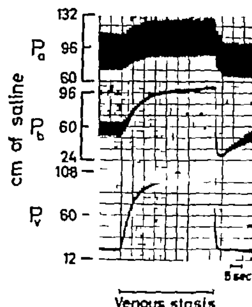


Fig. 5. Effect of occlusion of the venous drainage from the leg on the pressure in [illegible] small [illegible] outside the tibial bone (P [illegible]) on the tibial bone marrow pressure (P_b) [illegible] on the pressure in [illegible] small leg [illegible]tery (P_a). Not[illegible] that P [illegible] [illegible] P started [illegible] rise simultaneously.

present between the pressure in the small artery and the bone marrow pressure. During the further increase the venous pressure and the bone marrow pressure were equal. In this experiment the bone marrow pressure was high before the stasis was applied. When the bone marrow pressure was low before stasis, it was more markedly influenced by the rising venous pressure throughout the increase of the latter.

The variations in the bone marrow pressure as a result of variations in the arterial [illegible]essure were studied by stepwise occlusion and reopening of the femoral artery. [illegible]h step was maintained until the pressure in the small artery outside the bone [illegible]

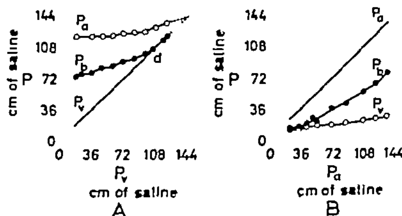


Fig. 6. *A* The bone marrow pressure (P[illegible]) and [illegible]he arterial press[illegible]re (P[illegible]) plotted [illegible] f[illegible] [illegible] of increasing venous pressure (P[illegible]) outside [illegible]he bone. Pressure values from [illegible] experiment of the type illustra[illegible]ed in Fig. 5. Not[illegible] tha[illegible] from *d* on, P and P [illegible]re equal. *B* The bone marrow pressure (P[illegible]) and the [illegible]enous pressure (P[illegible]) plotted as functions of [illegible] [illegible]epwise decrea[illegible]ing [illegible]rterial pressure (P[illegible]) in [illegible] small leg [illegible]rtery. Pressure [illegible] [illegible] from one experiment.

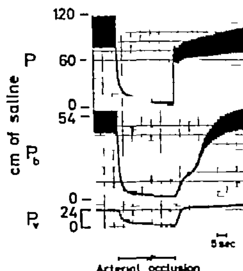


Fig 7 The effect of total occlusion of the arterial supply to the limb on the pressure in small leg artery (P_a) on the bone marrow pressure (P_b) and on the pressure in small leg ein outside the bone (P) The occlusion lasted for one minute.

well as the bone marrow pressure had reached stable values. Usually stabilization took place within 20 sec. Plotting of the bone marrow pressure against the arterial pressure gave curves which were con ex towards the arterial pressure axis (Fig 6 B) A high initial bone marrow pressure in a preparation with a normal arterial pressure was greatly influenced by changes in the arterial pressure, whereas an initially low bone marrow pressure at normal arterial pressure was much less influenced by such changes.

The effect on the bone marrow pressure of a total occlusion of the arterial supply to the limb for one minute is demonstrated in Fig 7 It should be noted that when the pressure became stabl during the occlusion, the bone marrow pressure was lower than the arterial pressure and equal to the venous pressure outside the bone. On reopening of the femoral artery the arterial pressure increased instantaneously and markedly whereas the bone marrow pressure increased more slowly and in two phases, a rapid rise without pulsations and a slower rise with increasing pulse pressure In some cases the bone marrow pressure increased moderately beyond its preocclusion level for a few minutes.

Comparison between the bone marrow pressure in different parts of the bone marrow cavity If the bone marrow cavity was opened to the atmosphere by drilling a hole with a diameter of 0.5 cm through the cortex, the bone marrow pressure fell to near zero in the opened region. In some distance from this region, however the bone marrow pressure often remained high (Fig 8 A) This reveals that the soft bone marrow tissue is able to carry a considerable pressure along its long axis. A comparison between the bone marrow pressures in different parts of the continuous bone marrow ca ty was therefore undertaken. The results were as follows 1) The bone marrow pressures recorded in different depths in the same region were always ery nearly equal.) The bone marrow pressure in the diaphysis and in the metaphyses was



Fig. 8. A Comparison between the bone marrow pressure (P) in the proximal metaphysis ( ) and the bone marrow pressure in diaphyseal region, which had been opened to the atmosphere by drilled hole which was 0.5 cm in diameter (b) B Recordings of the bone marrow pressure in different parts of the continuous tibial bone marrow cavity distal metaphysis ( ), middle of diaphysis (d) and proximal metaphysis ( ) The leg in horizontal position.

about the same in some cases, but often differences were present. In such cases the bone marrow pressure in the proximal metaphysis was usually but not always, the lowest one (Fig 8 B)

Discussion

The intramedullary venous pressure and tissue pressure Information about the normal intramedullary tissue pressure and about the factors which influence this pressure is important for the understanding of bonemarrow hemodynamics. It has been impossible to obtain direct reliable measurements of the normal intramedullary tissue pressure In this work the intramedullary tissue pressure has been examined indirectly through measurements of the intramedullary venous pressure This method is based on the assumption that the intramedullary veins are collapsible tubes. In trabecular bone the veins are possibly anchored to the bone trabeculae (Rutishauser *et al* 1954) but anatomical structures which suspend the veins within walled bone marrow cavities have not been described. Here the tissue pressure will exert its action directly on the collapsible veins, and the venous pressure must therefore be equal to or higher than the tissue pressure as the veins would otherwise collapse Furthermore, the intramedullary veins are tortuous endothelial tubes with a great volume capacity It seems unlikely therefore that their walls do carry any significant transmural pressure Consequently within smooth walled bone marrow cavities the intramedullary tissue pressure and venous pressure should be very nearly equal The intramedullary tissue pressure must then be relatively high, vary considerably and be pulsating in the same way as has been found for the intramedullary venous pressure The tissue pressure in the bone marrow is therefore in principle different from that in most other organs The conclusion that the intramedullary tissue pressure and venous pressure are about equal is strengthened by the finding that the intramedullary venous pressure was always equal to the bone marrow pressure in the same region, independent of whether the tip of the needle for bone marrow pressure measurement stood in the center or in the periphery of the cavity and independent of whether the procedures performed for measurement of the bone marrow pressure changed the intramedullary venous pressure or not. It should also be mentioned here that when Held and Thron (1962 a) tried to measure the intra

medullary tissue pressure by inserting a needle into the border region between the cortical bone and the bone marrow in the dog, they found a pressure between 10 and 20 mm of Hg which pulsated.

Since the regional intramedullary venous pressure and bone marrow pressure are equal, the latter must be some sort of a tissue pressure often, however it will not represent the normal tissue pressure, as the present experiments have shown that the procedures performed for measurement of the bone marrow pressure may cause the intramedullary venous pressure to increase considerably (Fig 2 B) It is reasonable to believe that this is due to the lesions made by the needle. Since these lesions were inaccessible for analysis, it has not been possible to relate the bone marrow pressure to the nature of the lesions. One peculiarity of such bone marrow lesions should be noted in this connection. In most organs injection of blood or other fluids into the tissue, with lesions also of the vessels, will lead to formation of a wound cavity from which the fluid is only slowly absorbed. In the bone marrow however blood or other fluids injected through a needle in the tissue, will immediately enter the venous system. Sometimes a visible wound cavity develops, sometimes not (Steinbach *et al.* 1957 von Süsse 1956)

The demonstrated effect of injury on the intramedullary venous pressure and thereby on the bone marrow circulation, should be taken into account also when other marrow-injuring methods are employed in circulatory studies.

In spite of the fact that the bone marrow pressure does often represent an abnormally high intramedullary tissue pressure, it is still a useful parameter in the study of several aspects of bone marrow circulation, as will be shown below

The blood pressure in the sinusoids and in the capillaries must in principle be somewhat higher than the tissue pressure and the pressure in the intramedullary veins. As, however the sinusoidal walls are unlikely to carry much pressure, and as the blood flow through the spacious sinusoids is slow (Brånemark 1959 Lamas, Amado and Da Costa 1946) the pressure difference between the sinusoidal blood and the surrounding tissue, is probably small. The capillary pressure is probably not much above the pressure in the sinusoids and as the tissue pressure is relatively high, there is probably only a small pressure difference across the capillary walls. This leads to the following view of the hemodynamics of the bone marrow circulation. The blood is distributed in the tissue by means of the arteries and arterioles. The pressure in these vessels must be greater than the tissue pressure, but the pressure difference across the vessel walls is less than in most other tissues In accordance with this the arterial marrow vessels have relatively thin walls (Yoffey 1965) Distant to the arterioles the blood oozes through the vascular channels, and the vascular pressures are no longer carried by the vessel walls but by the walls of the bone marrow cavity As far as the pressure relationships are concerned this cavity can therefore be considered as a part of the circulatory system, coupled in between the arterioles within the bone marrow cavity and the veins draining the bone.

These pressure relationships should be taken into account when the question is discussed of whether the sinusoidal walls are open or closed. They are also impor

tant in the discussion of the Starling mechanism for formation and absorption of interstitial fluid in the bone marrow tissue In this connection it should be noted that bone marrow tissue seems to lack lymphatic drainage (Yoffey and Courtice 1956 p 12)

Several factors will influence the regional intramedullary tissue pressure. Provided that the venous outflow from a region of the bone marrow is equal to the arterial inflow into the region, the intramedullary tissue pressure (P_m) is determined by the following simple equation

$Q = (P - P_m)/R_a = (P_m - P)/R$ i. e. $R_a/R_v = (P - P_m)/(P_m - P)$

(Q is flow P the systemic arterial pressure, P_v the normally low venous pressure in the great collecting veins from the limb R the arterial and R the venous resistance) The ratio between the resistances can be calculated in the various present experiments, since all three pressures are known. The absolute values of the resistances remain unknown, however since flow has not been measured. Normally the arterial resistance (R_a) has been considerably greater than the venous resistance (R) as the intramedullary tissue pressure was closer to the venous pressure (P) than to the arterial pressure (P) In some cases, however as e.g after injuries of the bone marrow the venous resistance achieved high values.

The pulsation in the intramedullary venous pressure is undoubtedly due to the arterial pulsation and the rigid enclosement of the tissue. The present experiments cannot tell however whether the pulse pressure is due to transverse conduction from the intramedullary arteries to the tissue surrounding them, or to a pulsating vis à fronte through the arteries and the arterioles on to the tissue

For the magnitude of the pulsations in the intramedullary tissue pressure the absolute and relative values of the resistances R and R are important This will, however not be dealt with here

The effect of noradrenaline and acetylcholine on the intramedullary venous pressure and tissue pressure Variations in the intramedullary venous pressure (P_{mv}) which is equal to the tissue pressure P_m) at constant systemic arterial (P) and venous pressure P are due to variations in the resistance ratio R_a/R As the intramedullary veins lack smooth muscle cells in their walls, variations in the vascular resistances caused by activity in vascular smooth muscles, can take place only on the arterial side of the system and in the veins outside the bone *Infusion of noradrenaline through the* femoral artery caused a great drop in the intramedullary venous pressure with a great decrease of it pulsations Fig 3 A) Stein Morgan and Porras (1958) and Bloomenthal, Olson and Necheles 1952) found a similar effect of noradrenaline on the bone marrow pressure in dogs. Herzig and Root (1959) observed a drop in the bone marrow pressure in the femur of cats upon stimulation of the sympathetic innervation to the bone Provided that noradrenaline and sympathetic stimulation do not exert a direct effect on the parenchymatous bone marrow tissue the most likely explanation for the drop in the intramedullary tissue pressure and its pulse pressure is that the resistance R increases due to constriction of arteries and arterioles. Any concomitant constriction of the veins outside the bone must have had a

less marked influence on the intramedullary venous pressure The effect of acetyl choline infusion through the femoral artery was a decrease in the ratio R_a/R_v (Fig 3 c) probably due to a dilatation of the arterioles.

Spontaneous changes in the systemic arterial pressure were often mirrored by similar changes n the intramedullary venous pressure. When noradrenaline was injected through an ear vein (Fig 3 B) a rise in the systemic arterial pressure occurred before noradrenaline reached the tibial bone marrow During this phase the three factors R_a, R_v and P ha e presumably remained about constant and the intramedullary venous pressure (P_{mv}) increased with the systemic arterial pressure (P) When noradrenalin reached the tibial bone marrow it exerted its local effects as it did when infused through the femoral artery

The relationship between the intramedullary tissue pressure and the arterial and venous pressure outside the bone The intramedullary venous pressure was higher than the pressure in the emissary vein outside the bone during free flow in the latter The venous resistance (R) from a region of the tibial bone marrow to the great collecting veins of the limb can therefore be regarded as consisting of two parts one part situated somewhere within the tibial bone, the other part situated in the small veins between the venous outlets from th bone and the great collecting eins of the limb As shown by Haddy *et al.* (1934) the latter resistance varies considerably and can often be great. It is not clear how and why the venous resistance inside the bone can show such marked ariations. When this resistance is small, the venous resistance outsid the bone dominates the situation, and the intramedullary venous pressure will passively follow the venous pressure n the surrounding soft tissues. This is, however not so when there is a great pressure difference between th intramedullary and the outside eins The question then arises whether a socalled water fall" phenomenon (Permutt, Bromberger Barnea and Bane 1962) may be present between the intramedullary and extramedullary veins. If that was the case, a rise n the enous pressure outside th bone would not be expected to influence the bone marrow pressure until the former pressure reached the level of the latter An increasing venous pressure outside the bone did, however influence the bone marrow pressure also when the former was considerably below the latter (Fig 5) A typical waterfall phenomenon is therefore not present

When the venous pressure outside the bone is increasing (Fig 6 A) the ratio $(P - P_m)/(P_m - P) = R_a/R$ increases towards infin ty (P now stands for the arterial pressure in the small arteries outside the bone, P_m is the bone marrow pressure, P stands for the venous pressure immediately outside the bone R_a for the resistance n the intramedullary arteries and R for the enous resistance between the bon marrow and the venous outlets from the bone.) This change can either be due to a decrease in the enous resistance an increase in the arterial resistance or to both.

Although a typical waterfall phenomenon was not present, t should be noted, that the bone marrow pressure nd the venous pressure outside the bone became equal when a considerabl difference was till present between the arterial pressure and the bone marrow pressure (t *d* in Fig 6 A) This means either that the venous

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bone marrow cavity In the rabbit tibia the proximal metaphysis has the greatest venous drainage capacity (de Marneffe 1951) and in accordance with this the bone marrow pressure was usually found to be lower in this than in other regions.

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the places for histamine and histidine. By comparing the fluorescent pots with standard spot of histamine its mount could be roughly estimated by the naked eye. When more tha 20 mg of tissue had been added small tail of histamine extending int th histidine spot sometimes was seen. W rarely dded more than 7.5 mg of fresh tissue to the plat

For fluorometric determinations 0.5—7.5 mg of fresh tissue was used. A histamine standard of 24 ng was alw ys run simultaneously as standard reference. T heck if all free histamine had left the tissue pieces, they were run second time bout 30 min later After this no more histamine could be detected I the organs examined with the exception of biopsies from the scalp and acne pustulae where 10—15 per cent of the original amount was found.

T study the influence of the weight of biopsies on the mount of histamine detected, biopsies from th same patient ranging from 0.5 to 20 mg were also run.

Histamine in tissue sections. Biopsies were immediately frozen in isopentane-filled tubes which have been chilled to —70 C in ethyl lcohol. The tissue is then mounted on cryostat chuck according t the method described by Pearse (1961) Sectioning is done in Pearse-Slee" cryostat t —20 C t 20 μ thickness. The sections are transferred on dry ice bed t a Pearse "Speedivac" Tissue Dryer (Edward High Vacuum Ltd., Crowly England) Freeze-drying is done in accordance t the instructions, i.e. two t five min. After freeze-drying th sections re stored in a petri dish.

About 10 to 15 sections are weighed on microbalance (0.5—1.0 mg) and placed i to the gel wells with 0.01 ml buffer

The advantage with the freeze-dry sections is that certain orga structures or diseased areas ca be cut out under dissection microscope (×40) for estimation of histamine. They can also be stained for histamine with orthophthaldialdehyde (Juhlin and Shelley 1966) and areas rich i histamine ca be nalyzed.

Results

The influence of the weight of biopsies on the amounts of histamine obtained appears in Table I It can be seen that from biopsies weighing less than 8 mg the values obtained are fairly constant

Table II shows histamine values per g fresh weight for various organs. In the stomach most of the histamine was found in the mucosa. Histological examination vealed histamine not only in mast cells and capillaries, but also diffusely distributed over all structures of the mucosa (Fig 2 a) Slightly more histamine was found at the base near the vessels but the variations were too great to draw ny definite conclusions. The highest histamine values were found in specimens from the body of the tomach near the great curvature. Two patients with gastric adenocarcinoma had an increased number of histaminecontaining mast cell in the border of the cancer nfiltration and 48 respectively 52 μg histamine/g fresh weight. Lower alues

T le I Influenc of biops weight on histamine con ent. The figures given show μg histamine base g fresh gh

Material	Biopsy weigh in mg				
	0 5 2	2 4	4—8	8—12	18—24
Skin (man 1		4 6	4 5	3 8	2 1
Ski (ma 2	3 (	3 1	9	2.0	1 1
Skin (m) 3	13	17 2	13 1	8 3	
Skin (ma 4	0	6	6 6	—	—
Lung (monkey)	67 6	60 0	53 1	—	30 8
Kidney cortex (monkey	1	1	2 3	1 6	0 9

TABLE II. Histamine content in various organs of man and vervet monkey (*Cercopithecus ethiops*)

Material		Number of subjects	Histamine base µg/g fresh tissue Range	Mean
Adrenal glands	Monkey	3	0.3— 0.8	0.5
Artery (femoral)	Monkey	2	1.3— 1.5	1.4
Colon	Monkey	3	3.1—12.2	6.2
Duodenum	Monkey	4	12 1—24.3	15.4
Heart	Monkey	4	1.0—10.0	6.3
Jejunum	Monkey	3	6.3—26.4	15.9
Kidney cortex	Monkey	6	1.5— 3.6	2 4
Kidney medulla	Monkey	6	0 — 0.9	0.7
Liver	Monkey	5	0.2— 1.8	1.0
Lung	Monkey	6	14.1—87 1	46.0
Lymphnode	Monkey	2	0.5— 1 1	0.8
Muscle	Monkey	3	0.8— 1 1	1.0
Pancreas	Monkey	3	0 — 3.3	1.2
Skin arm	Man	10	1 4— 5.6	4.0
Skin arm	Monkey	3	2.6— 8.5	5.1
Stomach mucosa	Man	4	4 1—30.1	15.1
Stomach mucosa	Monkey	5	2.0—24 1	11.3
Stomach muscularis	Man	4	0.9— 2.3	1 4
Stomach muscularis	Monkey	5	0.5— 1.8	1 1
Thyroid gland	Monkey	3	1 1— 4.1	2.3
Uterus	Monkey	4	6.8—16.4	12.6
Vein	Monkey	2	0	0

(3 and 8 ng) were seen in the middle of the cancer Specimens from the pylorus and cardia varied within wide limits and from the limited number of cases no definite conclusions can be drawn on the variations of histamine in different regions of the stomach.

The results from freeze-dry sections are given in Table III In the skin, most histamine was found in the capillary-rich papillae of the upper corium. The distribution of histamine is demonstrated in Fig 2 b. In the cerebrum of rat brain we obtained low concentrations of histamine With the staining method histamine was only seen along the small arteries especially in the cortex. When these vessel containing areas were cut out under magnification (× 40) and estimated for histamine between 0 5—1 5 µg/g dry weight was found In the part without visible vessels no histamine was obtained In the kidney the glomeruli showed a yellow fluorescence indicating histamine (Fig 2) From freeze-dry sections, the areas with glomeruli were cut out under dissection microscope They contained about two to three times more histamine than the inner part without glomeruli.



Fig 2. Freeze-dry sections stained with orthophthaldialdehyde for visualization of histamine
) Stomach (man) ×200. The mucosa (left) shows diffuse yellow staining I the muscular layer (right) some bright yellow mast cells are seen. (Freeze-dry method; Juhlin and Shelley 1966)
b) Distribution of histamine in skin ×180. (Incubation method; Juhlin 1966) Histamine yellow is seen in small vessels and mast cells. The epidermis is light blue
) Glomeruli containing histamine ppea bright yellow gainst blue background ×500 (Incubation method)

T BLE III Histamine content in freeze-dry sections.

Material	Number of specimens	Histamine base µg/g dry weight	
		Range	Mea
Ski (huma			
St. corneum nd gra ulosum	8	0	0
Basal cell la er nd papillae	8	9 —28	18
Corium	8	6 —14	10
Sebaceous glands	2	0 9— 1.3	1 1
Stomach (huma			
Mucosa	5	12.3—31.8	32
Muscularis	4	2 4— 6.1	4 1
Kidney (monkey)			
Cortex with glomeruli	4	4.8— 7.2	6 0
Medulla	4	1.0— 2.6	1 8
Brain (rat) cerebrum			
Area without essels	4	0	0
Area with vessels	4	0.7— 1.3	1 1

Discussion

The values obtained agree fairly well with most of those reported in the literature (Vugman and Rocha e Silva 1966 for ref.) It should be observed that several authors express the histamine content as µg g dried and defatted tissue which is not directly comparable with those from dried tissues. The values for skin histamine reported in the literature varies between 0 6—30 µg/g wet tissue (Lindell and Westling 1966)

We obtained in normal skin between 1 4—5.6 µg g histamine per gram wet weight, but only in certain skin diseases did we find higher values (Juhlin 1967) It therefore could be possible that some histamine is still bound in the skin and cannot be released by electrophoresis even after repeated freezing and thawing If less than 0.5 µg/g wet tissue remains, it can escape detection. I have tried to dry the biopsies at 100 C and also to add sulphuric acid before electrophoresis but have not been abel to find more histamine. In most methods used by others, histamine was extracted by boiling in strong acids. Schmiterlöw (1949) found that during such a procedure, histidine was transformed into histamine. This was avoided by extraction in alcohol which, when blood was examined, gave half as much histamine (Schmiterlöw 1949) The high histamine values found by some investigators might possibly be explained by such a mechanism.

Repeated freezing and thawing was found to be essential for histamine release. If the biopsies were just frozen once, only about 80 percent of the histamine is removed during electrophoresis. When the biopsies from the scalp and sebaceous glands of patients with acne were examined after repeated freezing a second electrophoresis of the biopsies showed that 10 to 15 per cent of the histamine remained. Since these areas contain sebaceous material, it seems probable that it might prevent the cell's destruction caused by freezing and, as a result, bind a certain amount of histamine or hinder its removal by electrophoresis. Subcutaneous fat, liver and the greasy material in atheroma contained very little histamine. That we did not find any histamine in a second electrophoresis can here be due to a failure to detect the small amounts of liberated histamine. It is possible, therefore, that when this method is used on tissue with a very high content of fat, it might give somewhat low histamine values.

The mast cells are known to produce and store histamine. For several organs a good correlation has been found between the number of mast cells and the histamine content of the tissue Riley and West 1953 It is uncertain to what extent we also have a free pool of non-mast cell histamin in the skin, such as that demonstrated in the stomach and salivary glands Brodie *et al.* 1966 and I y and Bachrach 1966 for ref.) According to Schayer 1963 histamine can also be synthesized in or near the vascular endothelial cells at a rate to maintain circulatory homeostasis. The yellow staining of the fin blood vessels with OPT Juhlin and Shelley 1966 Juhlin 1967) indicating histamine in the dermal papillae and the vessels of the brain upport this theory

In the kidney of monkey mast cells were sometimes seen around or between the glomeruli. They cannot however account for the higher histamine values found in these areas Most of the yellow fluorescence indicating histamine is seen in the capillaries of the glomeruli. Since 70 to 80 percent of injected C^{14} histamine is removed from the blood stream during its passage trough the kidney (Arnoldsson *et al.* 1962) one could speculate that it is taken up in the glomeruli. It is, however not necessary that a continuous uptake should explain the concentration of histamine in the glomeruli. It might also be due to a concentration of capillaries which are themselves rich in histamine.

Most of the histamine in the stomach was found in the mucosa and submucosa. Staining for histamine revealed a diffuse distribution in the mucosa. In subjects with a low histamine content it was mainly located in the blood vessels. Our results are in agreement with those of Murray and Wyllie (1964) who also have reviewed the literature on the distribution of histamine. However to get information of the role of histamine in gastric secretion it is necessary also to study the rate of histamine formation. The literature on this subject has recently been excellently reviewed by Code (1966)

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Transcapillary Fluid Absorption and Other Vascular Reactions in the Human Forearm during Reduction of the Circulating Blood Volume

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Abstract

MELLANDER, S and B ÖBERG *Transcapillary fluid absorption and other vascular reactions in the human forearm during reduction of the circulating blood volume* Acta physiol. scand. 1967 *71* 37—47

The responses of the human forearm vascular bed to reduction of circulating blood volume, produced by pooling about 600 to 800 ml of blood in the legs, were followed by plethysmographic technique The pattern of vascular response was similar to that previously found in anesthetized animals after hemorrhage. It involved reflex constriction of resistance and capacitance vessels and net transcapillary absorption of extravascular fluid. The capillary filtration coefficient averaged 0.0061 ml/min × 100 g tissue × mm Hg, and did not change significantly during the pooling of blood, indicating no major change of precapillary sphincter tone. The absorption of extravascular fluid can be related to fall in capillary hydrostatic pressure, which is, in turn, due to reflex increase of the pre- to postcapillary resistance ratio. This process seems to aid greatly in the restoration of plasma volume. A reflex resetting of the pre- to postcapillary resistance ratio might therefore constitute an important component in the overall cardiovascular compensatory adjustments to reduced circulating blood volume in man.

Studies on anesthetized experimental animals have shown that after hemorrhage there is a tendency toward restoration of plasma volume by means of a rapid transcapillary absorption of extravascular fluid, mainly from skeletal muscle and skin tissues into the circulatory system. This reaction is seen even in situations of unaltered arterial and venous pressures and can be ascribed to a fall in mean capillary hydrostatic pressure due to a reflex vasoconstriction which is relatively more pronounced in precapillary than in postcapillary resistance vessels (Öberg 1964 Lundgren, Lundvall and Mellander 1964) Such a reflex influence on the pre- to postcapillary resistance ratio and on capillary pressure in skeletal muscle has been suggested to be a fairly specific and potent mechanism controlling the fluid partition between the intra and extravascular compartments, and hence plasma volume (Öberg 1964)

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Even if it seems reasonable to assume that a similar mechanism for plasma volume restoration after hemorrhage is operating in man as well there are as yet no studies dealing with this particular problem. Extrapolation of data on such a subtle reflex adjustment obtained from experiments on anesthetized animals, to unanesthetized man might, at least from a quantitative point of view be quite misleading

The present investigation is an attempt to study directly the rate of net transcapillary fluid movement in normal healthy persons subjected to an acute reduction of the circulating blood volume. The investigation was performed on the forearm and permitted, *in addition an analysis of the simultaneous reactions in the resistance* capacitance and precapillary sphincter vessels.

Methods

The vascular adjustments in the human forearm were followed by means of plethysmographic technique modified from a method previously used for animals (Mellander 1960) and for the human foot (Mellander Öberg and Odelram 1964). By continuous recording of alterations in tissue volume it was possible to follow both the changes in regional blood content, reflecting the reactions of the capacitance vessels (abrupt and fairly rapid changes of tissue volume) and the [illegible] of net fluid movement across the capillaries (slower continuous changes of tissue volume) (for details see Mellander 1960 The reactions of the resistance vessels were assessed by recording arterial inflow pressure venous outflow pressure and regional blood flow Changes in the size of the capillary surface area available for exchange as determined by alterations of precapillary sphincter tone were followed by repeated determinations of the capillary filtration coefficient (CFC) *i.e.* ml fluid filtered across the capillaries per min, 100 g tissue and mm Hg transcapillary pressure gradient see Cobbold *et al.* 1963) CFC was calculated from the recorded rate of fluid filtration produced by elevating venous outflow pressure and hence mean capillary pressure a known amount. It was assumed that 80 per cent of the rise in venous pressure was transmitted to the capillary level Pappenheimer and Soto-Rivera 1948 Cobbold *et al.* 1963)

Experiments were performed on 12 healthy young males resting comfortably on a couch. Room temperature was 2[illegible] C The right forearm was enclosed in a waterfilled, temperature-regulated (34 C plethysmograph. Watertight closure was assured by a thin rubber sleeve which fitted the forearm smoothly but not tightly The plethysmograph was positioned so as to avoid any discomfort for the subject

Changes of forearm volume were recorded continuously with a volume transducer (Grass, PT [illegible] Forearm venous pressure was monitored from an indwelling catheter introduced through a superficial vein and advanced so far that its centrally directed tip was placed well inside the plethysmograph Venous pressure was measured with a Statham transducer (P 23 AC placed at the height of the upper water level in the plethysmograph Free communication through the catheter was [illegible] by [illegible] flushings with saline and heparin A cuff connected to a pressure bottle was placed around the upper arm proximal to the elbow Cuff pressure was measured with another [illegible] transducer [illegible] By [illegible] of the cuff [illegible] venous pressure could be raised to desired levels. Arterial blood pressure was measured in the left arm by auscultation Mean arterial blood pressure was taken as the diastolic pressure plus one third of pulse pressure Blood flow in the right arm was measured [illegible] by [illegible] plethysmograph During flow measurements [illegible] placed around the wrist and inflated to a pressure of 200 mm Hg to obstruct hand blood flow [illegible] from the pulse oscillations of the plethysmogram [illegible] venous pressure [illegible] cuff pressure and changes in forearm volume were recorded on a [illegible] recorder

The circulating blood volume in the main parts of the body was reduced by pooling blood in the lower extremities [illegible] placed as proximal as possible on both thighs, to 70 to 80 mm Hg The volume of blood pooled in the legs by this procedure [illegible] in the following [illegible] The amount of blood pooled per unit volume [illegible] at a cuff pressure of [illegible] 80 mm Hg was determined [illegible] by plethysmographic volume recording The figures were extrapolated to the total volumes of the legs the approximate length of which was determined [illegible] tically It was thus deduced that inflation of the thigh cuffs led to a reduction of the circulating blood in the main part of the body [illegible] from [illegible] 400 [illegible] 800 ml After cuff inflation the pooling of blood was completed in less than [illegible] the forearm [illegible] would be completed within one to one and a half min [illegible] the legs The procedure of pooling blood in the legs will below be referred to as [illegible] of reduced circulating blood volume

Experimental procedure. At the beginning of the experiment the cuff around the arm was inflated so as to elevate the forearm venous pressure well below the extravascular pressure exerted by the water in the plethysmograph. This was done to avoid the effects of the so-called waterfall phenomenon (cf. Kjellmer 1963) and to keep the veins somewhat distended so as to minimize passive pressure dependent adjustments of the veins (Öberg 1967). CFC was first determined repeatedly during resting conditions by increasing cuff pressure to a new steady level. After this, the two cuffs around the thighs were inflated in a standardized way for periods of 5 to 20 min, while forearm volume was continuously recorded. During the state of reduced circulating blood volume, CFC was again determined repeatedly and then, the thigh cuffs were deflated and the resting conditions restored. Finally blood flow and arterial blood pressure were measured repeatedly first at rest, and then during a period of standardized pooling of blood in the legs. This series of procedures was repeated two or more times in each subject.

At the end of the experiment, forearm volume was determined. According to Cooper Edholm and Mottram (1955) bone comprises 13 to 14 per cent of total forearm weight. The figures for tissue weight given below refer to soft tissue weight (bone excluded) of which muscle and tendons constitute about 80 per cent and skin and fat about 20 per cent. The capacitance response, i.e. the displacements of regional blood volume, are below expressed in per cent of the forearm blood volume at rest, which is assumed to be 2.5 per cent of the tissue weight. (cf. Mellander 1960)

Results

Fig. 1 shows the changes in forearm volume during a determination of CFC. In the control period (left) the arm cuff was inflated so as to raise forearm venous pressure to 20 mm Hg. The volume record is characterized by rapid regular oscillations synchronous with the pulse, by slower fairly regular oscillations synchronous with respiration, and by slow undulations of about 15—40 sec duration. These undulations are closely related to slight variations in venous pressure and reflect, in all probability spontaneous changes in capacitance vessel tone influencing the regional blood volume. In the course of the whole control period, there is a slow and continuous decline of forearm volume (see dashed line under volume curve) indicating a slow transcapillary absorption of extravascular fluid (cf. Mellander 1960).

For the determination of CFC, forearm venous pressure was elevated by further inflating the arm cuff. This led to a fairly rapid filling of the capacitance vessels, completed in about one min (volume pen repositioned 3 times) and amounting to roughly 7 ml, or 1.4 ml/100 g tissue. After this phase there was a further continuous, but slower increment of tissue volume (indicated by dashed line) due to a net

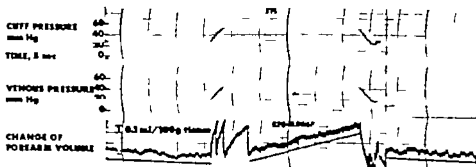


Fig. 1. Changes in human forearm volume, forearm venous pressure and arm cuff pressure during determination of CFC.

TABLE 1 Vascular responses in the human forearm to a reduction of the circulating blood volume The upper row shows mean values of 28 expts. on 12 subjects. The lower row shows the ranges of variation

Blood flow (ml min 100 g) Control	Blood flow (ml min 100 g) Pooling	Resistance response (% increase above control)	Capacitance response (% decrease of regional blood volume)	CFC (ml min × 100 g × mm Hg) Control	CFC (ml min × 100 g × mm Hg) Pooling	Change in rate of fluid absorption (ml min × 100 g)	Calculated fall of mean capillary pressure (mm Hg)
4 5	3.7	27	8	0,0061	0,0056	0,044	8
3.2—5 1	2.3—4 3	16—39	0—15	0,0035—0,0090	0,0035—0,0075	0 015—0 11	4,5—11

filtration of fluid from the capillaries into the tissues. The CFC, which in this case was 0 0067 ml min 100 g mm Hg, was calculated from this change in the rate of transcapillary fluid movement and the induced change in mean hydrostatic capillary pressure In the last part of Fig 1 venous pressure was restored to the control level which resulted in an initial emptying of blood from the capacitance vessels, and then a slow transcapillary fluid absorption at about the same rate as in the preceding control period

28 determinations of CFC were done in the 12 subjects. The mean value for forearm CFC at rest was 0 0061 ml min × 100 g × mm Hg and it ranged from 0 0035 to 0 0090 Table 1

From Fig 1 it might be noted that arm cuff pressure does not directly reflect forearm vein pressure In all experiments, cuff pressure exceeded vein pressure and this difference varied slightly between the subjects, possibly owing to the variable arm thickness

A reduction of the circulating blood volume produced by pooling some 600 to 800 ml of blood in the lower extremities, was associated with the following general pattern of response Heart rate increased slightly by some 5 to 10 per cent while as a rule mean arterial pressure and forearm venous pressure were insignificantly affected Forearm blood flow decreased by an average of about 25 per cent indicating a constriction of the resistance vessels. The flow resistance increased gradually until the pooling of blood in the legs was completed and it reached a steady state after one to one and a half min of thigh cuff inflation see Methods Forearm volume showed an initial, abrupt decrease co-ordinated in time with the development of the resistance response indicating a constriction of the capacitance vessel see below the magnitude of which varied greatly between the different subjects. When the capacitance vessel response was established tissue volume regularly showed a continuing slower decline at an approximately constant rate as long as the thigh cuff remained inflated As will be discussed below this volume change indicates a net transcapillary absorption of extravascular fluid into the circulatory system due

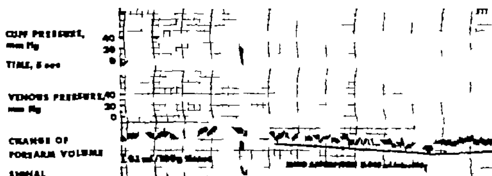


Fig. 2. Effects on human forearm volume and forearm enous pressure by reduction of the circulating blood volume accomplished by pooling bout 700 ml of blood in the legs (signal) Note the changed slope of the volume curve during the period of blood pooling indicating first, slight capacitance response and then, net transcapillary bsorption of extravascular fluid.

to a lowered mean capillary hydrostatic pressure. CFC determined during periods of blood pooling in the legs showed values a little below the control level, but the difference was probably not significant.

Table I summarizes the vascular responses in the forearm to a reduction of the circulating blood volume observed in 28 expts. on 12 subjects. Mean values as well as the ranges of variation are given.

Details concerning the changes in forearm volume following a reduction of the circulating volume can be seen from the original recording in Fig 2. In a preceding control period (left) forearm venous pressure was set at a relatively high level (30—32 mm Hg) which caused a slight net filtration of fluid at a constant rate (indicated by dashed line) At signal, the thigh cuffs were inflated so as to pool blood in the legs for a period of about 5 min. In this subject, this procedure elicited a change in arterial pressure from 120/70 to 115/75 mm Hg, an increase of heart rate from 56 to 60/min, and a reduction of forearm blood flow from 4.5 to 3 9 ml/min × 100 g tissue. All these adjustments were developed within one and a half min. Fig 2 shows that there was no significant change of venous pressure but a slight constriction of the capacitance essels (initial decrease of forearm olume) The magnitude of the capacitance response is most easily evaluated upon recovery after deflation of the thigh cuffs. This is probably due to the almost instantaneous restoration of blood volume that follows release of the thigh cuffs, contrasted to the much more gradual pooling of the blood, and hence more gradually developed cardiovascular reflexes, that accompany cuff inflation. The capacitance response produced in this case a regional blood volume displacement of about 0 08 to 0 1 ml/100 g tissue, or 3 to 4 per cent of total regional blood volume. When the constriction of the resistance and capacitance vessels had reached a steady state (some 60 to 90 sec after thigh cuff inflation) the forearm volume continued to decline at a constant rate (dashed line) indicating a net transcapillary absorption of extravascular fluid. From the observed change in volume slope the rate of absorption of extravascular was calculated to be of 0.042 ml/min × 100 g tissue. On release of the thigh cuffs vo-

attained after the capacitance response a slope similar to that in the preceding control period. Note that after recovery of the capacitance response the absolute volume of the forearm was reduced compared to the control volume just before blood pooling This difference corresponds closely to the amount of fluid absorbed.

CFC determined in the control periods averaged in this subject 0 0067 ml/min × 100 g mm Hg and it decreased to an average value of 0.0058 in the periods of standardized blood pooling It can be calculated that mean capillary hydrostatic pressure must have decreased by approximately 7 2 mm Hg (Is) to account for the recorded rate of fluid absorption in Fig. 2.

Discussion

In the present study a plethysmographic technique has been used for an in estigation of the vascular adjustments in the human forearm, following a reduction of the circulating blood volume The method has earlier been used and critically evaluated in many different types of animal experiments under strictly controlled experimental conditions e g Mellander 1960)

On the basis of such studies it is possible to interpret with reasonable accuracy the present results, despite the fact that some parameters could not be as strictly controlled in humans

The changes in forearm volume observed during a reduction in circulating blood volume mimic closely those seen in animal experiments during direct or reflex excitation of the vasoconstrictor fibres to e g the skeletal muscles (Mellander 1960, Öberg 1964 Lundgren et al 1964) The animal experiments have shown that this volume response consists of two components, an initial rapid volume decrease, due to constrict on of the capacitance vessels, and a subsequent, slower and continuous volume decline indicating an absorption of extravascula fluid due to a drop in capillary pressure in turn related to an increased pre to postcapillary resistance ratio These conclusions recei ed support from experiments in which, besides recordings of tissue olume changes, the capacitance response was simultaneously followed by recording the changes n regional blood olume by an isotope technique (^{51}Cr labelled ed cells see Ablad and Mellander 1963) and in which the rate of absorption of protein poor extravascular fluid from keletal muscle was followed by measurements of changes n plasma protein concentration in the venous effluent (Mellander 1960) Owing to the great resemblence between the observed changes in tissue volume in animals and man during reduced circulating blood olume there is every reason to bel e that the above interpretation of the volume curve is valid for the human forearm a well and that the observed changes permit a quantitative evaluation of both th capacitance response and the rate of extra ascular fluid absorption It would thus be erroneous to consider the continuous olume decline during the latter period of thigh cuff inflation (Fig 2) as a gradually increasing capacitance response sin e the resistance response reached a steady state within

90 sec after cuff inflation. If a gradually increasing vasomotor fibre discharge had occurred in whole this period, it should have been noted in the resistance response as well. Further forearm volume should then have been restored to the control level quite quickly when the capacitance vessels dilated again upon release of the thigh cuffs, but this did not occur After this dilator response of the capacitance vessels forearm volume was reduced below the control level, and the difference agreed very well with the calculated volume of fluid absorbed in the preceding period. Restoration of extravascular fluid volume after such a change is a process that can take a considerable period of time.

It is, moreover unlikely that the tissue volume changes recorded in the present study are due to variations in the lymph drainage from the region since the lymph vessels certainly must have been effectively obstructed by the inflated arm cuff.

The values for blood flow and CFC in the human forearm obtained at rest in the present study agree well with data reported by others (e.g Landis and Gibbon 1933 Cooper *et al.* 1955) The use of CFC for measuring changes in the size of the capillary surface area available for exchange, as affected by alterations in precapillary sphincter tone, has been described previously (Cobbold *et al.* 1963) As mentioned earlier CFC determination in the present study was based on the assumption that a given portion, or 80 per cent, of the venous pressure rise was transmitted to the capillaries. This assumption is not entirely correct, since alterations of the pre to postcapillary resistance ratio, resulting from reflex vascular adjustments, will change this figure. Thus, when the pre to postcapillary resistance ratio increases, as during blood pooling the capillary pressure will rise more for a given elevation of venous pressure and the CFC will consequently be somewhat overestimated. However as pointed out by Cobbold *et al.* (1963) the error of calculation, based on th assumption of a constant pre- to postcapillary resistance ratio will be quite small and will not preclud essentially correct conclusions concerning precapillary sphincter tone.

The observations made in this study during pooling of blood in the lower extremities ndicate that the vascular response pattern in human skeletal muscle and skin following a reduction of the circulating blood volume is qualitatively simila to that observed in skeletal muscles of anesthetized cats when exposed to acute hemorrhage (*e g* Öberg 1964) Thus, there is a constriction of the resistance and the capacitance essels, fai ly insignificant changes of precapillary sphincter tone (essentially un changed CFC) nd a net transcapillary bsorption of extravascular fluid resulting from fall in capillary hydrostatic pressure. This capillary pressure drop must, in turn be ascribed to a rise in the pre to postcapillary resistance ratio since it occurred in spite of unaltered arterial nd venous pressures.

The quant tative a pects of the vascula responses in the human forearm during blood pooling were shown in Table 1 These data ndicat that the magnitud of the responses of the resistance and, particularl the capacitance essels, were relati ly small. This agrees with the findings by Wood and Eckstein 1958 who observed that blood pooling in horisontall placed subjects onl occa ionall produced signs of venoconstriction

It is conceivable that all these vascular adjustments during blood pooling constitute a reflex response to the reduced circulating blood volume, elicited from various receptor sites, as is the case in cats (see Öberg 1964). Although in the present study the reduction of the circulating blood volume was accomplished without bleeding the subject there is every reason to believe that a similar pattern of response would be elicited during hemorrhage. The reflex did not seem to be evoked from local pain or pressure receptors since inflation of cuffs around both ankles to a pressure of 70 to 80 mm Hg (which hardly changes central blood volume) elicited no significant vascular reactions in the forearm.

When considering the compensatory reflex adjustments to hemorrhage, the rate of fluid absorption from human skeletal muscle and skin revealed by the present study (mean 0.044 ml/min × 100 g tissue) might, at first glance seem unimpressive. However both the time factor and the huge total mass of skeletal muscle and skin must be considered. Since total weight of these two tissues is about 35 to 40 kg in an adult 70 kg man the mentioned figure corresponds to a total net fluid absorption of 150 to 175 ml in a period no longer than 10 min. Some earlier studies indicate such a rapid restoration of plasma volume in man after hemorrhage. Thus, Kaufmann and Müller reported that a shed blood volume of 400 to 500 ml was 40 per cent restored within 15 to 20 min, a figure corresponding fairly closely to the one deduced above from the present experiments. It thus appears that this compensatory mechanism can play an important role for the plasma volume restoration in hemorrhage. The present study strongly suggests that the extravascular fluid absorption is a reflex vasomotor adjustment put into play even at fairly moderate changes in blood volume and before arterial pressure is significantly interfered with. It is thus not simply a passive event resulting from a passive fall in capillary pressure consequent to arterial hypotension even if such might reinforce the response.

It should be kept in mind however that the fluid entrance into the circulation is most rapid in the initial stages after a blood loss, and that it decelerates with time due to gradually decreasing reflex vasomotor fibre discharge following the refilling of the intravascular compartment and also possibly to a fall of plasma protein concentration. This replenishment of the circulatory system is therefore a self-limiting process which might produce no complete restoration of plasma volume as is evident from other studies (e.g. Ebert Stead and Gibson 1941). Despite this, the observed reflex resetting of the pre to postcapillary resistance ratio, which seems to be the main cause of the tissue fluid absorption must be considered a most important component in the overall compensatory cardiovascular adjustments following a reduction of blood volume in man. Animal experiments indicate that this particular mechanism is not operating in all tissues, for example not in intestine (Öberg 1964).

The amount of blood mobilized for the central circulation by reflex constriction of the forearm capacitance vessels during pooling of blood in the legs averaged 8 per cent of regional blood content. In absolute figures, this would correspond to some 70 to 80 ml of blood from the total skeletal muscle — skin mass. This volume is relatively small compared to the amount of fluid that can be made available for the circulation

by tissue fluid mobilization from skeletal muscle and skin during a prolonged period. However the capacitance response is an immediate adjustment and it is reasonable to assume that a substantial reflex mobilization of blood occurs from other vascular circuits as well, for example from the splanchnic organs. The capacitance response must therefore be a compensatory mechanism of great importance, particularly in the early stages of hemorrhage.

The maintenance of a virtually unchanged precapillary sphincter tone in hemorrhage, accomplished, in all probability by secondary metabolite dilatation opposing the reflex constriction (cf. Cobbold *et al.* 1963 Lundgren, Lundvall and Mellander 1964) might be beneficial from at least two points of view. The capillary flow distribution and the size of the capillary exchange surface are maintained fairly normal which aids in the exchange of nutrients from the reduced blood flow and in the transfer of extravascular fluid into the circulatory system.

The reduction of circulating blood volume, accomplished in this study by thigh cuff inflation, amounted to roughly 10 to 20 per cent of total blood volume. It is conceivable that a larger "blood loss" would lead to a corresponding increase of the sympathetic vasoconstrictor fibre discharge and an augmentation of the described vasomotor responses, including the fluid absorption. It should be noted, however that during very extensive hemorrhage, this normal reflex vascular response pattern might be changed both in a quantitative and a qualitative manner. Animal experiments have shown that, during prolonged arterial hypotension, the reflex constrictor response of especially the precapillary resistance vessels tends to deteriorate with time, while that of the postcapillary resistance vessels is longer maintained. This might, in later stages of shock, lead to a decrease of the pre- to postcapillary resistance ratio below normal, so that capillary pressure increases leading to a continuous loss of fluid from the circulatory system by ultrafiltration (Lundgren *et al.* 1964).

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Sympathetic Control of Consecutive Vascular Sections in Canine Subcutaneous Adipose Tissue

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Abstract

ÖBERG, B. and S. ROSELL. *Sympathetic control of consecutive vascular sections in canine subcutaneous adipose tissue.* Acta physiol. scand. 1967. *71.* 47—56.

The effects of electrical stimulation of sympathetic nerves to subcutaneous adipose tissue in the dog on the flow resistance, regional blood volume and the capillary filtration coefficient (CFC) were studied. The resting blood flow varied between 3.2—12 ml/min × 100 g and resting CFC between 0.02—0.035 ml/min × 100 g × mm Hg (mean = 0.027). The regional blood content was approximately 5 ml/100 g. Stimulation of the regional sympathetic nerves induced a pronounced decrease in blood flow, a two to threefold increase in CFC, a net transcapillary fluid absorption and decreased regional blood volume. Stimulation following administration of an α-blocking agent produced a blood flow increase up to 25—30 ml/min × 100 g, a small CFC increase and a small venous dilatation. These vascular responses were blocked by a β-blocking agent (propranolol). In comparison with skeletal muscle the vascular bed of subcutaneous adipose tissue was found to be well dimensioned for transcapillary exchange at comparable total blood flows. This was shown by the relatively high CFC values, which indicated a large capillary surface area and/or a high capillary permeability.

In previous papers (Orö, Rosell and Wallenberg 1965, Ngai, Rosell and Wallenberg 1966) sympathetic control of the blood flow in canine subcutaneous adipose tissue was reported. Electrical stimulation of sympathetic nerves induced a vasoconstriction the degree of which was related to the stimulus frequency. The vasoconstriction was blocked by dihydroergotamine and other sympatholytic agents with α-receptor blocking properties. Stimulation following the administration of these agents resulted in a blood flow increase which was also related to the stimulus frequency. This vasodilatation was blocked by β-receptor blocking drugs.

The results indicate that the subcutaneous adipose tissue is innervated by sympathetic nerves, the effects of which produce complex vascular reactions. In addition, activation of the sympathetic nerves to the adipose tissue also produces pronounced metabolic effects (see Havel 1965). Thus, the release rate of free fatty acids (FFA) was increased following stimulation with frequencies within the "physiological" range. This effect was blocked by β-receptor blocking agents (Rosell 1966, Fredholm

and Rosell 1967). It is reasonable to suggest that the vascular reactions in some way are functionally linked to the metabolic activity in the tissue. To evaluate this hypothesis it was found necessary to study the circulatory events following sympathetic nerve stimulation in more detail. Since mobilized FFA is transported from the fat cells across the capillary wall to the blood it is of primary interest to know not only changes in total blood flow but also alterations in capillary function. Furthermore adipose tissue amounts to some twenty percent of the total body mass. From a hemodynamic point of view it is therefore of interest to know how the transcapillary outward and inward filtration of fluid is controlled by the sympathetic nerves, and moreover how the blood volume (capacitance function) contained in the adipose tissue is regulated.

In order to carry out these studies canine subcutaneous adipose tissue was enclosed in a plethysmograph filled with Tyrode's solution. By recording total adipose tissue blood flow and tissue volume changes, the function of resistance, exchange and capacitance vessels was measured simultaneously (Mellander 1960).

A preliminary report of this study is presented elsewhere (Öberg and Rosell 1966).

Methods

Experiments were performed on 14 female mongrel dogs, anesthetized with sodium pentothal, 30 mg/kg bodyweight supplemented when necessary during the experiment. The trachea was cannulated. The inguinal subcutaneous adipose tissue, located between the pubis and mammary glands, was prepared on the right side according to the technique described by Rosell (1966). The adipose tissue was completely freed from the surrounding tissues except for the main artery, vein and nerve entering the adipose tissue via the inguinal canal. In some experiments the skin flap covering the subcutaneous adipose tissue was removed, but in many cases it was left in place. Since no quantitative or qualitative differences were observed between the two preparations as far as the vascular reactions were concerned, the results will be presented without mentioning the type of preparation used.

After administration of heparin the right femoral artery was cannulated and the blood was directed via a silicone filled drop recorder operating an ordinate writer (Lindgren 1958) to the artery supplying the adipose tissue. Intraarterial injections were given through a side-tubing in this exteriorized loop. The vein draining the tissues was likewise cannulated with a wide-bore plastic tubing, the free end of which could be placed at any desired level above the preparation. In this way the venous outflow pressure and hence the venous and capillary pressures in the adipose tissue, could be either varied or kept constant during the course of the experiment. The venous effluent was returned to the animal via a funnel connected to the left femoral vein. The adipose tissue was then placed in a plethysmograph with the artery, vein and nerve passing through a closely fitted opening which was filled with water-repellant grease (Plastibase Squibb) to make the plethysmograph watertight. Care was taken to avoid distortion of the structures passing through the opening. To avoid collapse of the vein when the plethysmograph was closed, the venous cannula was always advanced far enough into the vein so that the opening of the cannula lay well inside the plethysmograph. The plethysmograph was filled with Tyrode's solution kept at 38° C, and connected to a volume recorder which continuously registered tissue volume. Arterial blood pressure was monitored from the left femoral artery by means of a Statham pressure transducer and recorded on a Grass polygraph together with the blood flow. Tissue volume was recorded on a smoked drum.

In some experiments a constant flow technique earlier described in detail, was used (Renkin and Rosell 1962). The adipose tissue preparation was perfused from a blood reservoir capable of maintaining a constant flow perfusion despite great variations of flow resistance in the vascular bed. Shifts in resistance vessel tone were calculated from recordings of the perfusion pressure with a Statham pressure transducer. Electrical stimulation of the sympathetic fibres to the adipose tissue was performed with bipolar silver electrodes, placed either on the cut peripheral nerve just outside the plethysmograph (the central end of the nerve being cut) or on the cut lumbar sympathetic chain at the level of L_4. The sympathetic trunk was isolated by an anterior approach. The electrical stimuli were delivered from a Grass stimulator.

T obtain information concerning shifts in the size of capillary area available for exchange and/or changes in the permeability of the capillary membrane the capillary filtration coefficient (CFC) was repeatedly determined during the experiment. CFC is numerical alue for the filtrating capacity of the capillary membrane and denotes the amount of fluid filtered in ml/min and 100 g tissue for each mm Hg pressure difference across the membrane i ml/min × 100 g × mm Hg Determination of CFC was performed by sudden elevation of venous outflow pressure by known amount, usually 10 or 15 cm H_2O This maneuver led to rapid rise of tissue olume due t distension of the eins, followed by slower volume gain caused by filtration across the capillary membrane The rate of this filtration can be calculated and CFC deduced, provided the rise of mean capillary pressure is known I the present series of experiments 4/5 of the rise of enous outflow pressure was assumed to be transferred to the capillary level. For further details concerning the CFC determinations see Cobbold *et al* 1963 Folkow Lundgren and Wallentin 1963. Adipose tissue blood volume was calculated in some experiments. At the end of the experiment the artery was clamped nd the blood washed out with known amount of dextrane solution. The hematocrit was determined both in the wash out and in enous blood sample. From the degree of dilution of the blood by the injected solution the regional blood olume was calculated.

Results

The resting blood flow of the acutely denervated adipose tissue preparation *i.e* the alue found at the very beginning of the experiment before any nerve stimulation was given, varied in this series between 3.2—12 ml/min × 100 g, with a mean value of 6.5 ml/min × 100 g The corresponding value for CFC was 0 02—0 035 ml/mm × 100 g × mm Hg, the mean alue being 0 027 The regional blood content, as estimated at the end of the experiment, was approximately 5 ml/100 g

Fig 1 shows recordings from a typical experiment where the regional sympathetic fibres to the adipose tissue preparation were stimulated and the various ascular parameters followed. The blood flow prior to stimulation was high in this experiment, approximately 20 ml/min × 100 g a phenomenon which will be further discussed. Initially the venous outflow pressure was adjusted so that no significant change of tissue olume occurred, *i.e* an isovolumetric state. CFC was repeatedly determined by elevation of the venous outflow pressure by 10 cm H O a procedure which induced an initial rapid olume increase (venous distention) followed by a slower gain in tissue olume, which is nterpreted as a filtration (see dotted lines) The rat

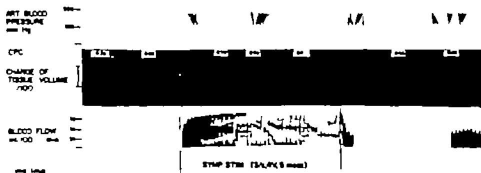


Fig 1 Effects of sympathetic nerv stimulation on blood flow tissue olume and CFC in he subcutaneous adipose tissue

of this filtration, correlated with tissue weight and deduced capillary pressure rise ga e a CFC of around 0 01 Stimulation of the regional sympathetic fibres produced a constriction of the resistance vessels reducing blood flow to one fifth of control. There was also a constriction of the capacitance vessels, as revealed by the rapid initial phase of tissue volume decrease This corresponded to a venous blood expulsion of approximately 1 7 ml/100 g which corresponded roughly to 1/5 of the blood content of the tissue. Following the initial rapid volume reduction there was a slower but continuous volume decrease occurring as long a the stimulation was given. This volume decline was ascribed to a *fluid absorption from the extravascular* spaces across the capillary membrane consequent to a reduction of mean capillary hydrostatic pressure The fall of capillary pressure implies that the precapillary *resistance increased relatively more than the postcapillary leading to a rise of the* pre postcapillary resistance ratio Repeated determinations during the stimulatory period showed that CFC was increased to roughly twice the initial value On cessation of the stimulation there was a dilatation of both the capacitance vessels, as evidenced by th volume increase and of the resistance vessels, which led to a blood flow increase well above the prestimulatory value This poststimulatory hyperemia often produced flows up to 2.—30 ml/min × 100 g which presumably represented a maximal dilatation of the resistance vessels. The period of hyperemia was usually long lasting 10—15 min, as evidenced by the high prestimulatory flow values shown in Fig. 1 These values represent a still lasting poststimulatory hyperemia from a stimulation performed approximately 12 min prior to the events demonstrated in the figure During the hyperemia CFC was somewhat elevated and returned to resting value concomitantly with the flow The experiment shown in Fig. 1 is typical from a qualitative point of view of all experiments where stimulation of the sympathetic fibres was performed without any receptor blockade The magnitude of the vascular responses were dependent upon the stimulation frequency Thus, at maximal stimulation (10—15 imp/sec) blood flow was reduced to 0.5—1 ml min 100 g blood mobilization amounted to 40—45 per cent of the total regional blood content and CFC increased to 0 1 or approximately three times the control value

Qualitati ely similar results were obtained irrespective of whether the stimulation was applied to the lumbar sympathetic trunk or to the supposedly mixed peripheral nerve. Essentially the results were similar when a constant flo perfusion technique was used, although the magnitude of changes in peripheral resistance, blood mobilization and CFC were somewhat maller The administration of a β-receptor blocking agent prior to stimulation did not affect the pattern of vascular responses except in one way The prolonged poststimulatory hyperemia did not occur and there was only a ery small and shortlasting flow increase after the stimulation was stopped.

The vascular responses to ympathet c stimulation after administration of an α-receptor blocking agent (dihydroergotamine) are illustrated in Fig 2. Here stimulation produced an almost maximal dilatation of the resistance vessels as indicated by the blood flow increase to 20—25 ml min × 100 g This dilatation

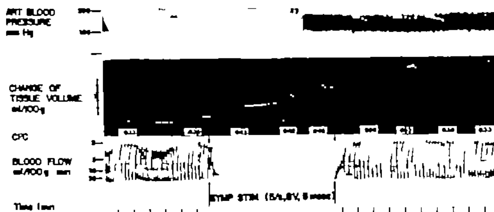


Fig. 2. Effects of sympathetic nerve stimulation on blood flow tissue volume and CFC in the subcutaneous adipose tissue. Prior t the recording 100 μg of dihydroergotamine was administered i.a.

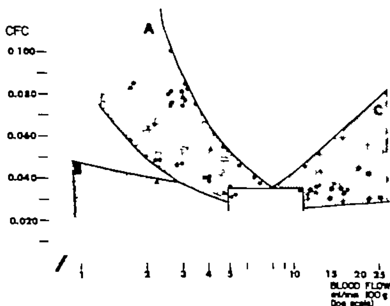


Fig 3 Relation between blood flow (ml mm 100 g and CFC (ml/min 100 g mm Hg) in 8 subcutaneous adipose tissue preparations during arious experimental procedures Open circles represent resting lues. V lues enclosed in area A represent sympa hetic stimulation with no receptor blockade or in some cases following β-receptor blockade V alues enclosed in area B represent mechanical restriction of blood flow V alues enclosed in area C represent poststimulatory hyperemia (small dots or during sympathetic stimulation following α-receptor blockade (large dots

appeared almost instantaneously with the start of stimulation and disappeared rapidly with the cessation of stimulation. There was only a slight initial volume increase, implying a very small venous dilatation. Moreover this was probably a passive phenomenon due to the rise of venous pressure when the precapillary resistance vessels dilated. This latter mechanism also led to a rise in the mean capillary pressure and a filtration of fluid into the extravascular spaces as indicated by the slow continuous rise of tissue volume during the stimulation period. CFC was found to increase somewhat both during and after the stimulation. All these vascular responses were completely abolished by the administration of a β-receptor blocking agent (propranolol). Qualitatively similar results were obtained in all stimulatory experiments after α-blockade (dihydroergotamine, phentolamine) irrespective of whether the stimulation was applied either to the sympathetic trunk in the lumbar region or to the mixed peripheral nerve or whether a constant flow or constant pressure technique was used.

Fig. 3 shows the relationship between blood flow and CFC as found during various experimental conditions in eight of the animals. The values within the white rectangle are those obtained at the beginning of the experiment prior to nerve stimulation and were considered to represent "resting" values. Thus, "resting" flow varied between 5 and 11 ml/min/100 g and CFC between 0.024 and 0.035. The dots enclosed in the dashed area (A) represent values obtained during sympathetic stimulation before any blocking agent was given, or in some cases, after a β-blocking drug. It can be seen that as the magnitude of the vasoconstriction increased the value for CFC rose to 0.1 ml/min/100 g/mm Hg at maximal vasoconstriction. The values indicated by the crosses (B) were obtained when the blood flow to the preparation was mechanically reduced by adjustments of a screw-clamp around the supplying artery. Evidently a flow reduction did not *per se* raise CFC significantly. The dotted area (C) represents values obtained during the poststimulatory hyperemic phase (small dots) or during stimulation after α-receptor blockade (large dots). In general it can be seen that an increased blood flow was followed by an increased CFC but CFC never reached the same high values as with stimulation before α-blockade.

Discussion

The present study is an extension of the investigations reported by Orö, Rosell and Wallenberg (1965) and Ngai, Rosell and Wallenberg (1966) concerning the sympathetic nervous control of blood flow in subcutaneous adipose tissue. In the present experiments, vasomotor fibre influence on other important vascular functions have been followed, i.e. the capillary exchange and the capacity functions in terms of changes in capillary filtration coefficient (CFC) and regional blood content, respectively.

The average resting values in acutely denervated subcutaneous adipose tissue for blood flow (6.5 ml/min × 100 g), for CFC (0.027 ml/min × 100 g × mm Hg) and for regional blood content (roughly 5 ml/100 g) are approximately 1.5 to 2 times those

found in resting skeletal muscle (*cf* Mellander 1960 Cobbold *et al.* 1963) indicating that the vascularization of adipose tissue is rather extensive (Hamberger and Widelitz 1963) in contrast to what is generally believed. It should be realized, however that adipose tissue may show varying degrees of metabolic activity and functional hyperemia" as suggested by the wide range of resting" flow values. Therefore the data may not represent true resting values and cannot be taken a priori as evidence that the subcutaneous adipose tissue is more richly vascularized than skeletal muscle. The fact that maximal flow capacity *i.e.* blood flow values found at maximal vasodilatation, amounts to only 25—30 ml/min × 100 g as compared with *50—70 ml/min × 100 g* in skeletal muscle indicates that the dimension of the resistance section is less in subcutaneous adipose tissue than in skeletal muscle. On the other hand the high CFC values f und particularly during sympathetic stimulation, and the relatively large blood content, suggest that the capillary and the postcapillary capacitance sections are of greater magnitude in subcutaneous adipose tissue than in skeletal muscle. The adipose tissue blood flow values found in this study are somewhat higher than those reported for innervated subcutaneous human adipose tissue by Larsen, Lassen and Quaade 1966) using a clearance method. *The discrepancy may be due either to methodological and species differ*encies, or to the fact that acutely denervated tissue was used in the present study.

Stimulation of the subcutaneous adipose tissue sympathetic fibres with supra maximal intensities produced vascular adjustments essentially similar to those found in skeletal muscle, *i.e.* constriction of resistance and capacitance vessels and a net transcapillary fluid absorption. However a clearcut difference was that in subcutaneous adipose tissue CFC increased markedly during stimulation, sometimes to values three times the control or in absolute figures to 0 1 ml/min × 100 g × mm Hg. This phenomenon seems to be unique and, therefore, necessitates further comment. CFC (see Methods) is a measure of the functional state of the capillary membrane and is, therefore related to both its permeability (the number and dimensions of the pores) and its size (the number of capillaries that at a given time are open to blood and hence available for exchange processes) Alterations of CFC do not provide information regarding which one of these two factors has changed. In other vascular beds the capillary permeability does not seem to be altered by vasomotor nerve activity (Cheng 1949 Folkow 1956) It is well known, however that in most vascular beds the vasomotor nerves regulate the number of capillaries open to flow by adjusting the tone of the precapillary sphincter vessels (*cf* Bücherl and Schwab 1952, Folkow and Mellander 1960, Rosell and Uvnäs 1962, Cobbold *et al* 1963) It is reasonable to suppose that this may be the case also in adipose tissue and that the marked increase of CFC during sympathetic activity may be ascribed to a relaxation of the precapillary sphincters In contrast sympathetic nerve stimulation in skeletal muscle skin and intestine primarily causes a closure of the sphincters and decrease of CFC (Folkow and Mellander 1960 Öberg 1964 Folkow Lundgren and Wallentin 1963 It is true however that in resting skeletal muscle CFC usually shows a slight secondary rise during prolonged sympathetic stimulation, often to

values above resting level This phenomenon has been ascribed to a sphincter relaxing influence of "unspecific metabolites" accumulated during flow reduction (Cobbold *et al* 1963) This secondary CFC rise in skeletal muscle is, however usually of a small magnitude and can not be compared with the two to threefold increase found in adipose tissue. Furthermore the CFC increase in adipose tissue does not seem to be due to accumulation of unspecific metabolites since CFC did not appreciably increase during a mechanically induced restriction of blood flow

It should be realized, however that stimulation of the sympathetic fibres to adipose tissue also induces profound changes in tissue metabolism as reflected by an increased rate of FFA release (Havel 1965 Rosell 1966) Therefore it is possible that the augmented metabolic activity in combination with the reduced flow during stimulation, causes a substantial accumulation of metabolites which may produce a pronounced relaxation of the precapillary sphincter section. A similar situation is seen in skeletal muscles when both the somatomotor and vasomotor fibres are stimulated simultaneously This procedure has been shown to increase CFC as much as three times the control level Kjellmer 1965 As a matter of fact, if the blood flow to the adipose tissue was kept constant during sympathetic stimulation, CFC did not rise to the high values found when there was a reduction of blood flow By the same token the CFC increase should be expected to be greatly reduced if the metabolic responses to sympathetic stimulation were blocked To test this a β-blocking agent (propranolol) was administered in amounts known to inhibit the FFA release. It was then found that the CFC increase was still of the same magnitude as following stimulation before the β-blockade Fig 3 This fact seems to indicate that the increased CFC is not related to the FFA release *per se* However the possibility remains that it is secondary to some other metabolic process not blocked by β-blocking agents.

It seems likely that the pronounced post-stimulatory hyperemia was due to metabolites accumulated in connection with the elevated rate of FFA release This was evident from the fact that when FFA release was inhibited by β-blocking agents the post stimulatory hyperemia completely disappeared.

Administration of α blocking agents to the adipose vascular bed abolished the vasoconstrictor responses to sympathetic nerve stimulation. Actually stimulation after the blockade induced an increases blood flow and the FFA release is potentiated Ngai Rosell and Wallenberg 1966, Fredholm and Rosell 1967 The blood flow often increased to 25 ml min × 100 g which corresponded to a maximal vasodilatation since administration of even large doses 10 μg min of various vasodilator drugs acetylcholine histamine etc did not further increase blood flow The question arises as to whether the vasodilatation is due to activation of specific vasodilator fibres or whether it is secondary to the increased metabolic activity in connection with the release of FFA following β-receptor stimulation. The fact that both the vasodilatation and the FFA release were inhibited by β-receptor blocking agents favours the latter hypothesis On the other hand, the rapid onset of the vasodilatation and, above all, its prompt cessation when stimulation stops does not seem to be compatible with a metabolic mechanism, since the response would be expected

to be more sluggish Thus, at present it is not possible to decide whether true adre nergic vasodilator fibres exist, or whether the vasodilatation is secondary to metabolic events, although the latter mechanism seems to be most likely The dilatation can not be due to antidromic stimulation of dorsal root fibres" since it also occurred when the sympathetic trunk in the lumbar region was stimulated. Furthermore, as has been reported earlier (Ngai Rosell and Wallenberg 1966) the vasodilatation was not due to a cholinergic mechanism since t was not blocked by atropine. The capacitance vessel response to stimulation after α-blockade consisted of a small dilata tion, which was probably only secondary to the resistance vessel dilatation. This implies that the vasodilatation is confined mainly to the precapillary section of the vascular bed. It is interesting t note that there was only a slight rise n CFC during sympathetic stimulation after α blockade. This may be due to the increased blood flow which counteracts an accumulation of metabol tes and, therefore, tends to diminish the dilating influence upon precapillary sphincters.

It was reported in the paper by Rosell (1966) that the vasomotor reactions to sympathetic nerve stimulation seem to interfere with the release of FFA, especially at high stimulation frequencies (5/sec, or more) The major part of the FFA liberation nt the blood took place at the end of and after the stimulation period, indicating that FFA may be trapped within the tissue. It was suggested that vasoconstrictor nerve activity might diminish the number of capillaries open to blood flow and therefore FFA could not be transported from the fat cells into venous blood until the vasoconstriction had passed. However this hypothesis is not supported by the present study which showed an increased CFC, and hence more favourable conditions for transcapillary exchange during sympathetic stimulation. It seems as if the func tional tate of the capillary membrane evaluated in terms of CFC, is not a lim ting factor for the release rate of FFA from the adipose tissue. This idea is further supported by the finding mentioned bove that after α-blockade a pronounced release of FFA occurs during stimulation despite a rather low CFC It thus looks as if th mpeded release rate of FFA during intense sympathetic stimulation is related to factors other than changes in the capillary surface area or capillary permeability The processes governing the transport of FFA from the fat cell into the blood must be tudied in more detail before the release mechanism can be precisely characterized

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Dissimilarities between the Central Control of Thirst and the Release of Antidiuretic Hormone (ADH)

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Abstract

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Effects of slow (7.5 μl/min) infusions of certain hypertonic solutions into the 3rd brain ventricle were studied in 4 unanesthetized goats. The only solutions found to elicit drinking were NaCl (0.85 M) and (to a minor degree) NaHCO (0.85 M) Other solutions tested (0.85 M solutions of Na-acetate and NH Cl, and 1 7 M solutions of urea and d-glucose) were negative in this respect. During 1 hr infusions of NaCl a strong cumulative drinking response was obtained in all animals within 3 to 8 min after the start of the infusion. No drinking occurred when the animals were hydrated prior to the infusion. The effects on water diuresis of short (2—10 min) periods of infusion of the same hypertonic solutions were studied in 3 goats after previous hydration. In 2 of the animals 4 min periods of infusion of the sodium salts and of NH Cl caused an inhibition of water diuresis of the neurohypophyseal type paralleling the effect of an injection of approximately 4 mU of ADH. A considerably smaller effect was obtained by urea and no inhibition of water diuresis was seen due to infusions of d-glucose. In one of the goats no inhibition of water diuresis was obtained from either NaCl or NH_4Cl. In this animal the site of infusion was more posterior and ventral in the 3rd ventricle than in the responding animals

Injections of small amounts of hypertonic NaCl solutions into the anterior hypothalamus or into the 3rd brain ventricle have been found to elicit polydipsia in the goat (Andersson 1953 Andersson Jobin and Olsson 1967) For this and other reasons it has been suggested that a hypothalamic osmoreceptor mechanism (similar to, or identical with that shown by Verney (1947) to control the secretion of ADH) may play an important role in the thirst mechanism.

However the effects of slow infusions of certain hypertonic solutions into the 3rd brain ventricle reported in this paper indicate that the two mechanisms may not be of an identical character

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Methods

Animal Three female goats (I II and III; b. wt 35 to 40 kg) nd one castrated male goat (IV b. wt .0 kg) were used. The animals were routinely onfined in metabolism cages by means of collars. All experiments wer conducted in these cages (Fig. 2) The goats were fed chopped timothy hay *ad lib* nd 400 g of commercial grain mix per day. They had free access to water. To guarantee dequat supply of N Cl they were given 6 g of N Cl dissolved in tepid tap water every after noon which the goats lways drank oluntarily

Cannulae stem T void th dead ntage of dead space" double cannulae system was used in the presen series of experiments.

An outer cannula (ex diameter 1.2 mm int diameter 0.8 mm) was permanently implanted into the 3rd brai entri le The outlet int the entricle was l th side of the cannula 0 5 mm from its entricula end (Fig 1) A short polyethylene tube was ttached t the upper free-end of the permanent annula and was losed with stainless steel stopper between experiments. During the infusion experiments, the topper was removed and a inner cannula, filled with the infusion solution and precisely fitting he permanent cannula, was inserted to the bottom of the permanent cannula (Fig 1 right)

Operation technique The implantation of the permanent outer cannula was mad under general anesthesia. A rectangular (10 8 mm) opening was made through the skull, exposing the midline of the brai Holes were drilled through the bone 2 mm from the midline nd about 3 mm from the nterior and he posterior borders of the opening These holes were drilled at an ngle of 45 through the dorsal tw thirds of the bone and int the opening A st inless steel wire was threaded hrough he holes and ts end were twisted together as show i Fig 1 (left) I addition, 3 or 4 dental root-screw were fixed into the bone close t the opening A cut was made through the dura in the midline By use of stereotaxic nstrument and under periodic X-ray control, the outer cannula was inserted through the cut in the dura nd downw rds int the 3rd entricle The position of the cannula opening the 3rd entricle was ascertained by X-ray control nd by observing the spon-

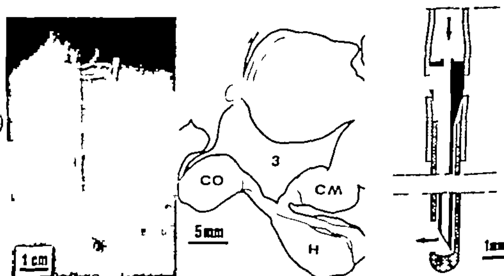


Fig. 1 An X pic re side iew of he kull of goa I showing he outer cannula permanently implanted int the 3rd bra en ricle with its outlet directed anteriorly The dental root-screws and the twisted men al wire referred in the ext re isible on the skull surface which, together with dental emen ansparen he X-rays ombined t mak long-lasting rigid fixation of the permanent cannul *Middle* A drawing of mid-sagi tal section through the diencephalon and the pituitary of he goa showing the position of the permanent cannula (*black*) i goats I and II. CO = Chiasma opticum CM = Corpus mammillare H = Hypophysis. 3 = the 3rd brain entricle *Right* The principle of the double-cannula system used for slow infusions int th 3rd entricle The inner cannula (*black* and the polyethylene tube connecting it with the infusion pparatus are filled with the infusion solution before it is inserted to the bot om of the outer permanent cannula (*dark grey*) I this manner the disadvantage of dead space is voided.

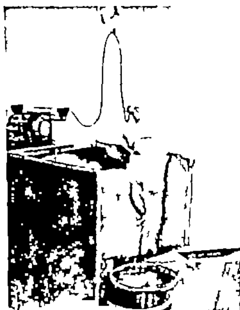


Fig. 2. The experimental rrangement during an infusion into the 3rd brain entricle of goat. The nimal remains without any extra restraint in the metabolism cage in which it is routinely confined. A polyethylene t be (tinted t show in the photograph) connects the inner 3rd entricular cannula syringe fitted onto perfusion pparatus. The tube is flexibly suspended by means of spring-balanced wheel and is taped one horn of the goat.

taneous outflow or inflow from the saline filled cannula. The short polyethylene t be t the top end of the cannula was closed with the stopper and the opening int the skull was filled with dental cement. Care was taken that the cemen covered the twisted stainless steel wire and the heads of the dental root-screws. I this ma ner rigid and long-lasting emplacement of be permanent cannula was btained.

Infusion technique Before inserting the inner cannula it was connected by means of long polyethylene be to 5 ml syringe fit ed onto perfusion pparatus (Unita/Perfusor) The entire ystem was filled with the infusion solution After insertion of the inner cannula the polyethylene be as taped to one horn of the animal nd was flexibly suspended by use of spring-balanced wheel (Fig 2) I this manner the animal retained its original freedom of movement and the infusion could be started or stopped without disturbing the goat.

H dration and urine ollection. A w er diuresis was established by giving he goats, by stomach t be about 100 ml of epid t p water per kg b w 60 90 min before urine collection started The urine was ollected in 5 10 or 15 min samples ia retention catheter inserted into the urinary bladder Urine N and K were determined by use of EEL flame photometer and urine Cl was determined according Brun (1949)

Results

A Thirst

1 *Sodium chloride* The infusions into the 3rd ventricle were made at a rate of 7.5 µl min and an 0 85 M NaCl solution was used. In the non hydr ted animals such infusions i variably caused a trong cumulati e drinking response The latency time the 3 female goats (I II and III was 3 to 4 min and in th larger castrated male goat (IV) 6 to 8 min. The mount drunk during 1 hr periods of infusion aried between 2.2 and 4 8 liters If a new infuson was started 10 min after a 1 h infusion a drinking response was usuall obtained again. Longer delays (20 or 30 min) between the 1 h period nd a subsequent shorter infusion period generally did not esult in ny further drinking (Fig 3

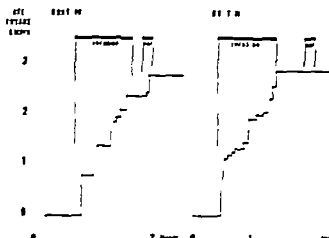


Fig. 3. Cumulative drinking responses of goats IV and II to slow (7.5 µl/min) infusions of 0.85 M NaCl solution into the 3rd brain ventricle.

During similar infusions of NaCl made to test the ADH release following hydration, no drinking occurred.

The 1 h infusions of NaCl in all animals caused the same conspicuous (up to 10 times) increase in urinary excretion of Na and of Cl as that observed earlier as a consequence of repeated 3rd ventricular injection of hypertonic NaCl solutions (Andersson, Jobin and Olsson 1966).

2. *Sodium bicarbonate.* The drinking response to infusions of $NaHCO_3$ (0.85 M, 7.5 µl/min) was studied in goats II, III and IV when not hydrated. A definite drinking response was obtained, but the latency time before onset of drinking was considerably longer than during similar infusions of NaCl and the amount of water drunk was less than one third of that consumed during the equivalent infusion of NaCl. However, the infusions of $NaHCO_3$ had an excitatory and tremor-inducing effect which was not seen during the infusions of hypertonic NaCl.

3. *Other substances.* Long term (20 to 60 min) infusions (7.5 µl/min) of 0.85 M sodium acetate or NH_4Cl and of 1.7 M urea or d-glucose were repeatedly made in all 4 animals when not hydrated. These infusions caused no drinking.

B. Release of ADH

The 3 female goats I, II and III were also used following hydration to study the effect of short term (2 to 10 min) infusions (7.5 µl/min) on water diuresis. The same solutions which were used for studies of the drinking response were employed.

Infusions of 0.85 M *NaCl* in goats I and II revealed that a 4 min period of infusion caused an inhibition of water diuresis and a rise in urinary electrolyte concentration of the same order as that obtained after the i.v. injection of 4 mU of ADH (Pituitrin, Park, Davis & Co.). Two min periods of infusion of NaCl did not cause any significant reduction in urine flow, whereas a 6 min period gave a somewhat more pronounced effect than the 4 min period (Fig. 4, upper left). Four min periods of infusion of 0.85 M *Na-acetate* and *$NaHCO_3$* caused an inhibition of water diuresis of the same magnitude as a 4 min period of NaCl infusion (Fig. 4, right below).

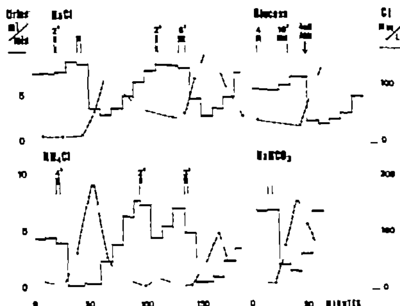


Fig 4 *Effects on water diuresis of short-term infusions into the anterior part of the 3rd ventricle of goat II* The goat was given 100 ml of tepid wa er per kg b wt. by stomach tube, 60 to 90 min before the start of each of the experiments. *Upper left:* A sequence of 2, 4 2 and 6 min infusions of 0.85 M NaCl t rate of 7.5 μl/min. Note that the 4 min period causes an inhibition of water diuresis and rise in urinary Cl concentration similar to that obtained after an i injection of 4 mU ADH (upper right) The 2 min periods are not sufficient to cause ny apparent release of ADH. *Lower left* A similar sequence of infusions of 0.85 M NH Cl performed at the same ra e. An even more pronounced inhibitory effect is seen than due to the N Cl infusions. *Upper right* Four nd 10 min periods of infusion (7.5 μl/min) of 1 7 M d-glucose which do not cause any apparent release of ADH. The subsequent injection of 4 mU ADH (Pituitrin, Park, Davis & Co") causes an inhibition of w ter diuresis of the same order as that seen due t 4 min periods of sodium salt infusions. *Lower right:* The effect of 4 min period of infusion (7 5 μl/min) of 0.85 M $NaHCO_3$.

Unexpectedly NH Cl which was not found to cause drinking in the non hydrated animal, was even more effective in eliciting an nhibition of water diuresis than was N Cl. Two and 3 min periods of infusion of 0 85 M NH Cl at the usual rate (7.5 μl/min) were sufficient to cause an obvious inhibition of water diuresis and a rise in urinary electrolyte concentration (Fig 4 left below)

Urea (1 7 M) on the other hand, had to be infused for 8 min or more to cause an inhibition of water d uresis and 1 7 M *d-glucose* (even when infused for 10 min or more) was completel negative in this respect (Fig 4 upper right)

Goat III which in th non-hydrated state responded to NaCl with cumula tive drinking differed from goats I and II Th usual 4 min periods of infusion of 0.85 M NaCl of NH Cl did not influence th water diuresis. Even 10 min periods of infusion of NH Cl were negati e, and the same was the case when the rat of infusion of 0 85 M NaCl was ncreased from the usual 7.5 μl/min to 15 and 20 μl min for 8 min periods How ver the response of this animal to ADH was normal The only obvious experimental difference between the animals was that the site of infusion in the 3rd ventricle was more dorsal and more anterior in goats I nd

II (as in Fig. 1 middle) than in goat III. In goat III the ventricular end of the permanent cannula was placed in the ventral part of the 3rd ventricle at a transverse plane through the infundibulum.

Discussion

Verney's (1947) fundamental studies in the unanesthetized dog have provided a basis for the understanding of the mechanism responsible for the control of the release of ADH from the neurohypophysis. The secretion of ADH seems to be mediated by "osmoreceptors" located in the anterior hypothalamus and/or the anteromedial region of the thalamus (Jewell and Verney 1957). The "osmoreceptors" are apparently not stimulated by a rise in total body fluid osmolarity *per se* but rather by a change in the extracellular fluid which reduces the cell volume (such as might be caused by an elevation of extracellular Na concentration). Thus Verney (1947) showed that intracarotid infusions of hypertonic sodium salts and sucrose effectively elicit a release of ADH, whereas similar infusions of urea do not cause any release of ADH. Verney suggested that the reason for this difference may be that urea diffuses relatively rapidly into the cells and therefore does not act as an effective stimulus to the "osmoreceptors".

In a recent series of experiments (Andersson *et al.* 1967) the influence of 3rd ventricular injections of 0.1 ml of certain hypertonic solutions on water intake was studied in the goat. It was found that neither 0.85 M NH Cl nor 1.7 M d-glucose elicited drinking, whereas 0.85 M NaCl was very effective in this respect. In contrast to the injections into the 3rd brain ventricle, injections of this amount of hypertonic NaCl into the lateral ventricle did not cause drinking. These findings were taken as further evidence for the concept that hypothalamic "osmoreceptors" in Verney's sense might be concerned not only with the release of ADH, but also with the development of the urge to drink. It was thought that NH Cl and d-glucose might have diffused more readily into the cells than NaCl, and would therefore not act as an effective stimulus to the "osmoreceptors".

The idea that the "osmoreceptor" mechanism for ADH release and the central "thirst" mechanism are of identical character needs to be reconsidered as a consequence of the present study. Recent experiments on bulk flow and diffusion in the cerebrospinal fluid system of the goat (Pappenheimer *et al.* 1962, Heisey, Held and Pappenheimer 1962) provide much valuable information which may serve as a basis for such a reconsideration. In these studies it has been shown that normal bulk flow in the cerebrospinal fluid system of the goat averages 0.16 ml/min. Since the flow is in an anterior to posterior direction it is probable that very little of the solutions infused in the present experiments (7.5 µl/min) spread anterior to the site of infusion. Heisey *et al.* (1962) have further shown that the ventricular wall of the goat is permeable to urea, Na and to a minor degree fructose. It may be expected that the ventricular wall permeability to d-glucose is considerably higher than for fructose in this animal, since this is the case in the rabbit (Bradbury and Davson 1964).

Bradbury and Davson found d-glucose to be transported out of the ventricles at an even higher rate than urea. Although no study of ventricular wall permeability to NH_4Cl has been found in the literature, it is reasonable to conclude that the variations in the effects of substances used in the present experiments were not due to differences in their rate of penetration of the ventricular wall.

In goats I and II short term infusions of the hypertonic sodium salts and of NH_4Cl effectively inhibited water diuresis and caused a simultaneous rise in urinary solute concentration paralleling that seen after an i. v. injection of ADH (Fig 4) In all probability this effect was due to a release of ADH from the neurohypophysis. Equally hypertonic urea was much less effective and no inhibition of water diuresis occurred after similar infusions of d-glucose. It can not be taken for granted that the apparent release of ADH in goats I and II was due to a specific stimulation of osmoreceptors in Verney's sense. It is of interest, however to compare the results of the present study with those following intracarotid infusions in the dog (Verney 1947) Verney has not studied the effect of hypertonic NH_4Cl, but he obtained the same effect with intracarotid infusions of sodium salts as that seen after 3rd ventricular infusions of these salts in goats I and II At variance with the results in these goats are the results of intracarotid infusions of d-glucose and urea in the dog When infused intracarotidly urea causes no release of ADH in the dog whereas d-glucose is effective, although to a lesser degree than sodium salts and sucrose A possible explanation for the discrepancy between these studies may relate to the different routes the infusions take to the responsive part of the brain. During intracarotid infusions the infusion approaches the brain from the vascular side of the blood-brain barrier In contrast intraventricular infusions by-pass this barrier

If one assumes that the hypertonic NH_4Cl released ADH by stimulation of an osmoreceptor mechanism in Verney sense, the thirst caused by infusion of NaCl into the 3rd ventricle does not seem to be elicited by the same mechanism. The 3rd ventricular infusions of NH_4Cl inhibited the water diuresis in goats I and II even more effectively than did corresponding infusions of NaCl (Fig. 4) Still infusions of NH_4Cl in the same animals when not hydrated failed to induce drinking in spite of the fact that the goats were not visibly disturbed by the infusions. It appears that the drinking effect in the present study was due to a stimulation of nervous elements rather specifically sensitive to an elevated NaCl concentration of the internal environment, although these elements also responded to a minor degree to $NaHCO_3$. There thus seems to exist certain characteristic differences between the "thirst eliciting" elements and the "osmoreceptors" regulating the secretion of ADH

The results obtained in goat III may indicate that there also is a spatial separation between the osmoreceptors and the central thirst mechanism. In this animal a thirst effect was seen during infusions of hypertonic NaCl into the 3rd ventricle, but identical or even more intense infusions did not cause any inhibition of water diuresis when the animal was hydrated The site of infusion in this goat was more posterior and ventral than in goats I and II It has previously been shown by electrical stimulation that there exists an overlapping between the regions in the hypothalamus

from which a release of ADH and thirst may be elicited in the goat (Andersson and McCann 1955). The "thirst" region extends further posterior. The studies of Jewell and Verney (1957) also indicate that the antero-medial thalamus plays a crucial role in the neurohypophysial "osmoreceptor" mechanism. Further experiments involving infusions at different sites in the 3rd ventricle in the same animal are planned in order to give a more conclusive answer to the question as to whether the "osmoreceptors" controlling the ADH release and the "thirst-eliciting" elements are spatially separated.

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The Effects of CO and Hydrogen Ions on Active Na Transport in the Isolated Frog Skin

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Abstract

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The effect of pH on the active sodium transport of the isolated frog skin has been investigated. The sodium transport has previously been considered to be particularly sensitive to lowering of pH in the solution bathing the corium side of the skin. However when pH was lowered by unilateral titration with carbon dioxide, the active sodium transport was more sensitive to changes of pH on the epidermal side than on the corium side of the skin. Raising bicarbonate concentration from 2.4 to 57.5 or 115 mM increased the tolerance to carbon dioxide This effect of bicarbonate was exerted from the corium side of the skin. The differing responses seen in the presence of 2.4 57.5, and 115 mM bicarbonate showed no direct correlation to pH of the media. When phosphate concentration was below 1 mM, lowering of pH values between 6.5 and 5.8 with HCl or H PO in the medium on the corium side had only slight effect on the sodium transport. At phosphate concentrations of 5—6 mM the transport was found to be inhibited rapidly The results suggest that the active sodium transport of the isolated frog skin is affected by conditions lowering the intracellular pH of the transporting cells

Available information uniformly indicates that the active sodium transport of the isolated frog skin is directly affected by changes of the hydrogen ion activity in the media bathing the two sides of the skin (Schoffeniels 1955 Snell and M Intyre 1960) The present work shows that the hydrogen ion activities of the media cannot be solely responsible for the effects on short circuit current, which have so far been ascribed to be a result of ambient pH It is demonstrated that the reactions of the frog skin t low pH values are markedly dependent on the way in which the pH change is brought about. The consequences of reducing pH by increasing carbon dioxide partial pressure (pCO_2) or by adding HCl or NaH PO to the media are thus all different.

Methods

The abdominal skin of *Rana temporaria* was dissected off and mounted as flat sheet between conical lucite chambers. The area of skin exposed was 7 1 cm The chambers and the auxiliary equipment employed, as well as the technique of short circuiting the skin, were adopted from Ussing and

Zerahn (1951) with a few minor modifications mentioned below. Short circuit current and potential difference across the skin were measured directly. Results obtained on skins displaying an initial short circuit current less than 100 μA were discarded. DC resistance was calculated by dividing the open circuit potential difference with the short circuit current per unit area.

Titration with CO was always carried out in only one of the two chambers at a time. Thus it was possible to discriminate between the effects of CO when applied either from the epidermal or from the corium side of the skin. pH-stating by CO_2 was carried out as follows. The electrolyte solutions bathing the skin were continuously aerated and circulated by atmospheric air. Appropriate amounts of CO_2 were applied from a side tube guarded by a magnetic valve (Danfoss EVJD 6). pH of the electrolyte medium was continuously recorded by means of glass and calomel electrodes and fed to a titrator (Radiometer TTT_{1c}). The titrator operating the magnetic valve of the CO_2 inlet, drew the amount of CO required to adjust pH to preset value.

The electrolyte solutions employed were modifications of the ordinary frog Ringer's solution in which different fractions of sodium chloride and sodium bicarbonate could be interchanged. The composition of the ordinary Ringer solution was as follows: Na^+ 115 mM, K^+ 2 mM, Ca^{++} 1 mM, Cl^- 116.6 mM, HCO_3^- 2.4 mM. This medium is referred to in the following text as "2.4 mM bicarbonate medium". The so-called 57.5 mM and 115 mM bicarbonate media employed were of the same composition as the above, except that 55.1 and 112.6 mM chloride had been replaced by equimolar concentrations of bicarbonate. In the experiment depicted in Fig. 4 and 5 we employed a bicarbonate free medium, 119 mM chloride being the only anion present ("119 mM chloride medium").

The relation between pCO_2 and pH values was calculated from the equation

$$pCO_2 = HCO_3^- / \alpha \cdot \text{antilog}\ (pH - pK')$$

in which pCO_2 is the partial pressure of carbon dioxide (mm Hg), HCO_3^- is the bicarbonate concentration (mM), α is the millimolar absorption coefficient of CO_2 (0.043 mM/mm Hg, Tp. 25° C) and pK' is the negative logarithm of the first apparent dissociation exponent of carbonic acid.

The mean value of pK' calculated from our results of simultaneous determinations of pH and pCO_2 of bicarbonate solutions (ionic strength 0.115, Tp. 25° C) was 6.196 (S.E. 0.005; n = 20). This is in agreement with a value of 6.193 which was calculated from a pK_1 of 6.37 (Hodgman 1963) applying a correction for the ionic strength (μ) of the solutions $pK_1' = pK_1 - 0.512\sqrt{\mu}$ (Fitzsimmons and Sendroy 1961). The phosphate buffers used as pH standards were in accordance with the scale of the National Bureau of Standards (Bates 1954).

Unidirectional fluxes of Na were measured in short circuited skins using ^{22}Na. The radioactive tracer was added to one side of the skin, and after an initial equilibrium period of 15—30 min samples were removed from both sides every 30—60 min. The isotope was counted in a γ-well scintillation counter. Counting times were extended sufficiently to reduce statistical errors of counting rates below one per cent.

Results

I. Effect of unilateral variations of pCO

a) 2.4 mM bicarbonate medium

The technique of varying pCO separately on the epidermal and on the corium side of the skin rendered new information about the reactions to carbon dioxide. Fig. 1 shows an experiment in which both sides of the skin were bathed in the ordinary Ringer containing 2.4 mM HCO_3^-. This experiment displays several characteristic features of the CO_2 effects. On increasing outside pCO_2 from 0.3 to 1 mm Hg (period 2) short circuit current rose by approximately 10 per cent, accompanied by a small decrease of electrical resistance (from 1100 to 1014 ohm cm²). When pCO_2 was further increased to 40 mm Hg (period 4) the current immediately dropped to 9 per cent of its initial value although a pH of 6.34 on the epidermal side has often been reported not to affect the frog skin. By further stepwise increase of pCO_2 to 80 mm Hg (period 5—6) current was almost abolished. The electrical resistance of the skin simultaneously rose to 3400 ohm cm². When in the following period

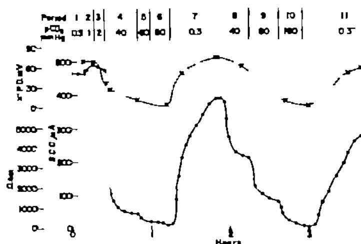


Fig. 1 The effect of unilateral CO_2 application on short circuit current (µA/7 1 cm²) potential difference × (mV) and electrical resistance O (ohm cm²) of a frog skin mounted in 2.4 mM HCO_3^- medium. In the periods 2—6 CO_2 was applied to the epidermal side, in the periods 8—10 to the corium side of the skin. The pCO_2 values (mm Hg) in the titrated chamber are shown in the figure. The pCO_2 in the nontitrated chamber was 0.5 mm Hg.

pCO_2 of 0.5 mm Hg was reestablished (period 7) current and potential difference returned to values close to those found at the start of the experiment. Now conditions were reversed, pCO_2 being increased stepwise on the corium side of the skin. The short circuit current was again clearly affected by CO_2, but to a smaller degree than previously Elevation of pCO_2 to 40 mm Hg (period 8) only caused a reduction of the current to 55 per cent of its initial value, and reduction below 10 per cent of initial value was not achieved until pCO had been increased to a value of 160 mm Hg (pH 5 74) (period 10) The reduction of short circuit current was accompanied by an increase of electrical resistance to 2916 ohm cm On withdrawal of CO_2 from the electrolyte medium all parameters returned towards initial values.

Stimulation of the short circuit current by CO could only be evoked from the epidermal side. Such a stimulation was observed in all but two of 17 skins bathed in 2 4 mM HCO- medium. The average maximal increase of current was 12 per cent (S E. 2 per cent) and the average pCO at maximal stimulation was 2.5 mm Hg

The main observation illustrated by Fig 1 is that the sodium transport of the skin is more sensitive to elevation of pCO in the solution bathing the epidermal side than in the solution on the corium side of the skin. Collecting results from 17 skins it was found that the current dropped off with a slope of about 60 per cent reduction t a tenfold increase of pCO

Table I summarizes the results from 11 skins in which we determined the pCO_2 required to reduce the short circuit current below 10 per cent of its initial value In the external medium the average value of pCO was 67 mm Hg (pH 6 14) In contradistinction an average of 228 mm Hg pH 5 62) was required, when CO was applied from the corium side of the skin

TABLE I Mean and S. E. (in brackets) of the pCO_2 values and of the pH values required to reduce the short circuit current more than 90 per cent of the initial value in the presence of the 2.4 mM bicarbonate medium. Titration with CO_2 was carried out on one side of the skin, the pCO_2 of the medium in the opposite chamber was below 1 mm Hg (n = number of experiments)

	Medium HCO_3^- mM	n	pCO_2 mm Hg	pH
CO_2 titration in external chamber	2.4	11	67 (7)	6.14 (0.05)
CO_2 titration in internal chamber	2.4	11	228 (32)	5.62 (0.05)

b) *Effects of CO_2 in the presence of 57.5 or 115 mM bicarbonate*

Table II shows the average pCO_2 values required to reduce the short circuit current below 10 % of its initial values when skins were bathed in 57.5 or 115 mM bicarbonate media. It is evident that the effect of CO_2 was modified when the bicarbonate concentration was increased. 90 per cent reduction of short circuit current was achieved at average pCO_2 values of 293 and 503 mm Hg respectively when pCO_2 was increased on the epidermal side of skins bathed by 57.5 or 115 mM HCO_3^- media. These partial pressures should be compared to a value of 67 mm Hg, which was found in the 2.4 mM HCO_3^- medium (Table I). An extreme tolerance to elevation of pCO_2 on the corium side was manifested by skins bathed in the 115 mM bicarbonate solution. Even by increasing pCO_2 to 700 mm Hg (which was the maximum obtainable) short circuit current could not be abolished. The average percentual current remaining at this pCO_2 was 40 per cent (n = 5). A similar tendency was noted in the presence of 57.5 mM HCO_3^-. In these experiments the short circuit current was reduced to an average of 25 per cent of the initial value at a pCO_2 of 700 mm Hg (Table II).

Fig. 2 shows the course of a typical experiment. When the skin was mounted in 2.4 mM bicarbonate medium the short circuit current could be completely abolished at a pCO_2 of 80 mm Hg (pH 6.04) with application of CO_2 to the epidermal side (period 1). On withdrawal of CO_2, current and potential difference returned to initial values. After the lapse of 85 min the media on both sides of the skin were replaced by 57.5 mM HCO_3^- medium. It may be noted that half of the chloride could be replaced by bicarbonate without any pronounced changes of current or electrical potential although as a general rule electrical resistance was found to decrease with increasing bicarbonate concentration (cf section d). In period 4—7 pCO_2 in the medium on the external side of the skin was increased stepwise. It was now found that a pCO_2 of 360 mm Hg (pH 6.77) was required to abolish the short circuit current. Following recovery the corium side of the skin was exposed to a pCO_2 of 700 mm Hg (pH 6.48, period 9). It appears that the immediate drop of

TABLE II Effect of CO on short circuit current t bicarbonate concentrations of 57.5 and 115 mM. The values stated for the titration with CO_2 in the external chamber are the mean and S. E. of the pCO_2 required to reduce short circuit current more than 90 per cent of initial value. The results stated for the titration with CO_2 in th internal chamber are the verage currents remaining at pCO_2 of 700 mm Hg in the internal solution. (= number of experiments)

	Medium HCO- mM		pCO_2 mm Hg	pH	Short circuit current remaining (per cent of initial value)
CO_2 titration in external chamber	57.5	7	293 (34)	6.88 (0.03)	<10
	115	7	503 (43)	6.93 (0.04)	<10
CO_2 titration in internal chamber	57.5	5	700	6.48	25 (range 8—36)
	115	5	700	6.78	40 (range 21—60)

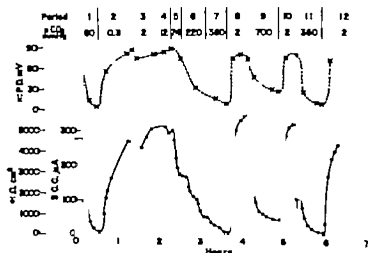


Fig . The effect of unilateral CO_2 titration on the short circuit current (μA/7 1 cm²) potential difference (mV) and electrical resistance (ohm cm²) of frog skin. Both bathing solutions were initially the 2 4 mM bicarbonate medium. Before period 3 both solutions were replaced by 57.5 mM medium. CO titration was carried out on the epidermal side in all periods except in period 9 and 10. The pCO_2 values in the titrated chamber are indicated on the figure. The pCO in the nontitrated chamber was 2 mm Hg

short circuit current in this period leveled off at a value of 45 μA, which was 15 per cent of the initial value. In the subsequent period carbon dioxide was again applied to the external sid of th skin, repeating the finding that the short circuit current vanished at a pCO of 360 mm Hg



Fig 3. The effect of unilateral titration with CO on the short circuit current (μA/7 1 cm²) potential difference × (mV) and electrical resistance O (ohm cm) of a frog skin bathed with 2.4 mM bicarbonat medium on the epidermal side and with 115 mM bicarbonat medium on the corium side. CO_2 was pplied to the epidermal side of the skin. The pCO_2 values in the external chamber re shown on the figure The pCO in the nontitrated hamber was 2.5 mm Hg.

The qualitative reactions of the current to CO_2 were similar whether the skin was bathed in 2.4 57 5 or 115 mM bicarbonate media. The skins were—without exceptions—most sensitive to the application of CO from the epidermal side. It was also noted that stimulation of the current could only be effected by CO_2 on the external side of the skin. Such a stimulation was observed in 4 of 5 skins mounted in 57.5, and in 6 of 7 skins mounted in 115 mM bicarbonate media. The pCO_2 causing stimulation was higher in the 57 5 and 115 mM bicarbonate media (range 9—50 mm Hg) than in the 2 4 mM bicarbonate medium.

c) *The effect of carbon dioxide when bicarbonate concentration is increased unilaterally*

In another series of experiments we examined the effect of CO_2 when bicarbonate concentration was increased on one side of the skin only The results clearly indicated that the bicarbonate concentration in the medium bathing the corium side of the skin is determinant for the differing tolerances to CO_2 which were demonstrated in the preceding paragraph

The reaction to CO was examined in 5 skins which were mounted with 115 mM bicarbonate medium bathing the epidermal side and with the 2 4 mM bicarbonate medium in the nternal chamber The average pCO_2 required in the internal medium to reduce short circuit current below 10 per cent of its initial value was 230 mm Hg (S E. 12 n=5) This value must be compared with the value of 228 mm Hg (Table I), which was found when skins were bathed in 2.4 mM HCO_3^- medium on both sides.

Fig 3 shows the reactions of a skin which was bathed th 115 mM bicarbonate medium on the corium side and w th 2 4 mM bicarbonate medium on the external

TABLE III Mean values and ranges of short circuit current (SCC) and electrical resistance before and during titration with CO_2. Results from 47 expts. Frog skins were mounted in media with varying bicarbonate and chloride concentrations. The short circuit current during exposure to CO was calculated in per cent of the values found in the equilibration period preceding CO_2 titration. In th experiments in which short circuit current could not be reduced below *10 per cent of initial value, the percentual short circuit* current reported is that measured at pCO, of 700 mm Hg (Ranges are stated in brackets)

Medium HCO^- mM	CO_2 pplied to	SCC (μA/7 1 cm²) before titration (= 100 per cent)	D. C. Resistance (ohm cm²) before titration	Minimum SCC (per cen of initial value)	D. C. Resistance (ohm cm²) at minimum SCC
2.4	epidermis	255 (115—540)	1,695 (812—2,880)	10	4 119 (1,695—7,988)
2.4	corium	252 (105—410)	1,631 (606—4,369)	10	3,344 (1,521—9 407)
57.5	epidermis	319 (166—430)	1,372 (788—2,737)	10	5,460 (2,169—10,650)
57.5	corium	309 (245—350)	1 448 (1,210—1,822)	25	3,284 (1 790—5,486)
115	epidermis	394 (108—620)	1 161 (744—1,692)	10	3,628 (1,650—5,237)
115	corium	329 (181—700)	1 116 (573—2,148)	40	2,424 (1,389—3,807)

side pCO was increased stepwise on the epidermal side of the skin. Reduction of *short circuit current below 10 of its initial value* was attained at a pCO_2 of 500 mm Hg (pH 5.25 period 6) This is close to the average value of 503 mm Hg found for skins in the 115 mM bicarbonate medium (Table II) Also in agreement with the values of Table II 30—40 of the initial current remained when pCO_2 was increased to 700 mm Hg on the corium side of the skin. (This part of the experiment is not shown in the figure.)

It is thus apparent that the maintenance of short circuit current which has been demonstrated at high pCO_2 levels is related to the bicarbonate concentration on the corium side of the skin. The possible contribution of diffusion of anions to the short circuit current, when bicarbonate and chloride concentrations differ on the two sides of the skin, is considered in paragraph 5, dealing with the relation of sodium fluxes to short circuit current.

d) *The electric resistance and the short circuit current.*

In Tabl III we have compiled data on changes of resistance following exposure of kins to carbon dioxide It wa a fundamental characteristic that decrease of short circuit current which was effected by CO_2 was accompanied by increased resistance. This increase was independent of the medium employed, and was found whether

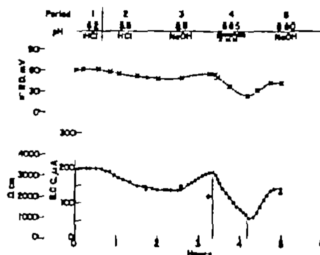


Fig 4 Differing effect of HCl and of phosphate on short circuit current (μA/7 1 cm²) potential difference × (mV) and electric resistance O (ohm cm²) of frog skin. Phosphate concentration and pH of the medium in the internal chamber are shown on the figure. Both bathing solutions were the 119 mM chloride media. (For further explanation see text)

CO was applied to the epidermal or to the corium side of the skin. In all experiments in which current could be reduced below 10 % of its initial value the electrical resistance increased by 200—300 per cent.

By inspection of the values of resistance found at a short circuit current of 100 per cent, another feature appears from Table III namely a tendency of resistance to decrease and of the short circuit current to increase with increasing bicarbonate concentration of the medium. Thus the resistance of skins bathed by 115 mM HCO⁻ medium (1116—1161 ohm cm) was only 70 per cent of the resistance found in the presence of the 2.4 mM HCO⁻ medium (1631—1695 ohm cm²) The average values of short circuit current in the 115 mM HCO⁻ medium were 1.3—1.5 times higher than those found in the presence of the 2 4 mM HCO⁻ medium.

2 The effect of HCl and of H_3PO_4 on the short circuit current

The reaction of the short circuit current to lowering of pH in the Ringer solution on the corium side of the skin was dependent on the phosphate concentration. Fig 4 shows an experiment in which pH of the medium in the internal chamber was lowered by addition of hydrochloric acid. Initially pH was lowered to 6.2 without any effect on the short circuit current during a period of half an hour In period 2 pH was further reduced to 5 8. The response was a slow reduction of the short circuit current to about 75 per cent of its initial value. When pH was changed to 8 8 by titration with NaOH the current slowly reverted to its initial value (period 3) Following recovery a pH of 5 85 was produced by adding 2 mM NaH_2PO_4 This was followed by a marked reduction of the short circuit current which after 30 min was 33 per cent of initial value (period 4) On titration with NaOH the current returned towards initial value.

The importance of the phosphate concentration for the rate of reaction of the short circuit current is further demonstrated by the experiment shown in Fig 5.

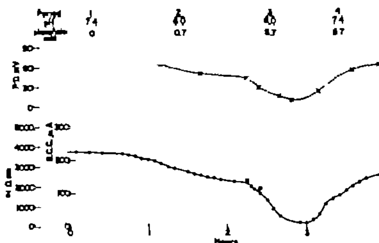


Fig 5. Effect of phosphate concentration on short circuit current (μA/7 1 cm²), potential difference × (mV) and electric resistance O (ohm cm²) of frog skin t pH 6 on the internal solution. The phosphate concentration and pH of the medium in the internal chamber are shown on the figure. Both bathing solutions were the 119 mM chloride media.

Lowering pH in the unbuffered 119 mM chloride medium from 7 4 to 6 0 in the internal chamber by addition of H PO induced a slow fall in the short circuit current from 220 to 130 μA over a period of 100 min. The DC resistance remained unchanged in this period. The phosphate concentration of the Ringer solution was 0 7 mM. At the start of period 3 the phosphate concentration was increased to 5 7 mM without changing pH. In 35 min the short circuit current dropped from 130 to 16 μA and the DC resistance increased from 2,200 to 4 000 ohm cm By titration with N OH the pH was changed to 7 4 and the short circuit current in creased to 142 μA in 50 min. In other experiments in which phosphate concentration of 5 mM caused a similar rapid fall in short circuit current as shown in Fig 5 we found only small or no changes in the current when phosphate concentration was below 0.5 mM at pH values between 5.3 and 6.0.

3. Relation of the short circuit current to unidirectional Na fluxes

Under a majority of conditions the short circuit current of the frog skin has been found to equal net sodium transport (Ussing and Zerahn 1951) Our experiments showed that this relation also holds when skins bathed in Ringer solutions w th high bicarbonate concentrations are exposed to the effects of carbon dioxide. The current of sodium influx exceeded the short circuit current by 5—10 per cent in all three media. The average ratio

(current of Na influx/short circuit current)

n the presence of 2.4 mM HCO^- was 1 06 (S E. 0 02, n=8) in 57.5 mM HCO^- 1 11 (S E. 0.03 n=6) and in the presence of 115 mM HCO^- 1 07 (S.E. 0 02, n=6)

The sodium influx remaining when short circuit current was completely abolished by increasing pCO in the 2 4 mM HCO^- (7 3 mCoulomb cm⁻² h⁻ S.E. 2.5

$n=4$) was equal to the value of sodium efflux found by direct measurement (6.3 mCoulomb cm^{-2} h^{-1} S.E. 1.3 $n=10$). Compared to the latter value sodium efflux was significantly increased when skins were bathed in 115 mM HCO_3^- medium, the average being 23.1 mCoulomb cm^{-2} h^{-1} (S.E. 1.7 $n=19$). In both media sodium efflux was unaffected by changes of pCO_2.

Differing concentrations of chloride and of bicarbonate in the media on the two sides of the skin might create current of anion diffusion breaking the equality between short circuit current and net sodium transfer. In four experiments where the bicarbonate concentration was increased on one side of the skin only (as reported in section 1 c) it was found that the current of Na influx was between 94 and 101 per cent of short circuit current, no matter whether the high bicarbonate was on the epidermal or on the corium side of the skin.

Discussion

Previous examinations of the effect of carbon dioxide on the sodium transport have been carried out with a technique implying simultaneous variations of pCO_2 on both sides of the skin (Ussing 1948, Linderholm 1952, Snell and Mc Intyre 1960). Consequently the differing reactions of the sodium transport following unilateral exposure to CO_2 have escaped attention. Schoffeniels (1955) and Snell and Mc Intyre (1960) employed Ringer solutions buffered with fixed concentrations of phosphate (5 and 6 mM respectively), thereby diverting attention from the fact that the pH response is modified by the phosphate concentration as evidenced by Fig. 4 and 5. Coincidentally the response of the frog skin to a pCO_2 of 35—40 mm Hg, as was applied by Snell and Mc Intyre to Ringer solutions with a bicarbonate concentration of 2.5—20 mM, almost perfectly mimicked the effect on the short circuit current of changing pH from 7.1 to 6.5 in a medium containing 6 mM phosphate.

There is a similarity in the effects of phosphate and of bicarbonate in the fact that both anions exert their effect on sodium transport from the corium side of the skin. Increased tolerance to CO_2 is found when bicarbonate concentration of the medium is raised (Table I and II). However, only an increase of bicarbonate concentration on the corium side of the skin has this protecting effect on short circuit current (Fig. 3). Skins bathed in a medium of high bicarbonate concentration on the external side only did not increase their tolerance to CO_2, even though they were kept in the medium for several hours. Phosphate causes a reversible decrease of short circuit current when pH is lowered below 8 in the internal solution. Since the pK_2 of phosphoric acid at 25° C is 7.21 (Hodgman 1963), it is likely that a sufficient concentration of monovalent phosphate ions is necessary for the effect of phosphate to develop rapidly. Apparently bicarbonate and phosphate cannot reach their sensitive sites from the solution bathing the outside of the skin. Assymmetrical distribution of endogenous lactate about the frog skin was noted by Leaf and Renshaw (1957), who found more lactate accumulating in the fluid bathing the inside of the skin, than in the solution bathing the epithelial side. Leaf (1959) further showed that the permeability of the epithelial side of the toad bladder to lactate was some 15-fold

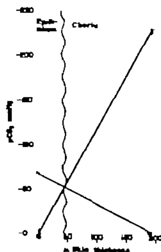


Fig. 6. Schematical representation of the carbon dioxide gradients, sufficing to eliminate 90 per cent of short circuit current when CO_2 is applied to the epidermal side or corium side of the frog skin bathed in 2.4 mM bicarbonate media. (For further explanation see text.)

less than the permeability of the serosal surface. A similar asymmetrical permeability of the frog skin to the anions bicarbonate and primary phosphate might prevent these ions from reaching their site of action from the epidermal side of the skin. Whether this site is located in the interspaces between the cells, or whether it is located intracellularly cannot be determined from the present experimental evidence. However our findings are compatible with the hypothesis that short circuit current is affected by conditions lowering intracellular pH of the transporting cells. The rapid effects of CO are thus understandable when the high permeability of the frog skin towards this gas is considered (Wright 1934) If the cells responsible for active transport of Na are located in the epithelial layer of the skin, it is natural that the effect of identical CO_2 partial pressures is most marked when carbon dioxide is applied from the epidermal side of the skin. If pCO_2 is increased unilaterally and the CO_2 gradient through the skin is assumed to be linear the value of pCO causing elimination of short circuit current may be evaluated by the method illustrated by Fig 7 Linear gradients were drawn from the average values of pCO found to reduce short circuit current more than 90 % in the 2.4 mM HCO⁻ medium (Table I) As the mode of action of carbon dioxide is thought to be identical whether CO is applied to the internal or the external side of the skin, the point of intersection of the two gradients is taken to indicate the actual pCO operating at the CO sensitive site as well as the relative location of that site in the skin. Employing the results of Table I we found that the short circuit current was reduced more than 90 per cent, when a pCO of about 50 mm Hg was operating at a site which is located about one fifth of total skin thickness from the cornified cells of the epithelial layer Thickness of epithelium and corium was measured on histological preparations of 22 skins employed in this study Average skin thickness was 193 μ (S.E. 10) and the average thickness of the epithelium was 45 μ (S.E. 3) These results suggest that CO exerts its action in the deep layers of the epithelium, although it must be admitted that there are many assumptions involved in the above considerations.

The slow reaction of the short circuit current following a lowering of pH in the internal medium by adding small amounts of HCl or H PO can be understood if the cells have a low permeability to H A greater permeability to HCO- and to H_2PO^- might enable these buffer anions to interfere more rapidly with intracellular pH

Mullins (1958) showed that the equality of net sodium current and of short circuit current is broken, when a skin is bathed in Ringer solutions containing anions with differing mobility in the skin (e g Cl and I) A similar condition was found not to arise in the experiments in which chloride and bicarbonate concentrations on the two sides of the skin differed. The agreement found between the currents indicates that there is no great difference between the rates of penetration for chloride and bicarbonate through the frog skin.

The average values of DC resistance and of short circuit current before titration with CO_2 show a tendency of the current to increase and of the resistance to decrease, when increasing fractions of chloride in the media are replaced by bicarbonate (Table III)

A possible explanation for the tendency of short circuit current to increase in the presence of bicarbonate might be that the bicarbonate ions cause an increased influx of Na into the sodium transporting cells, secondarily increasing active sodium transport through the skin. This mechanism would be similar to the one suggested to underlie the increased sodium transport rates observed in skins of rana esculenta in the presence of quaternary amines (Skou and Zerahn 1959)

Our thanks are due to dr Claus Brun, head of the Central Clinical Laboratory for provision of the histological preparations of the frog skins employed in this study

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The Negative Inotropic Effect of Vagal Stimulation on the Heart Ventricles of the Duck

By

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Abstract

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Experiments on anesthetized ducks show that the contractility of the heart ventricles of this diving species can be powerfully depressed by the vagal nerves. This vagal negative inotropic effect shows the same characteristic frequency-response relationship as the vagal negative chronotropic effect and may at high "physiological" discharge rates, reduce contractile force to less than half the control. This vagal negative inotropic influence, as combined with the reflex inhibition of the sympathetic positive inotropic influence, that also occurs when awake ducks dive, can reduce stroke volume below control despite the often profound rise in central venous pressure seen during submersion (Folkow Nilsson and Yonce 1967).

In a recent paper (Folkow Nilsson and Yonce 1967) it was shown that ducks during submersion often display a most profound reduction of cardiac output at a relatively constant arterial blood pressure. This output reduction, sometimes to less than 5 per cent of resting cardiac output, was usually even more pronounced than the typical bradycardia of the diving reflex. Therefore the stroke volume appears, if anything to be reduced during the dive.

Considering the fact that central venous pressure usually increases considerably during submersion, from approximately atmosphere pressure up to 10—20 mm Hg it must be concluded that the ventricles during the dive are exposed to a considerable negative inotropic influence. If this were not the case stroke volume should increase according to the Frank-Starling relationship between diastolic pressure and myocardial contractile force. Though it is well known that the bradycardia during submersion is predominantly a consequence of a reflex increase of vagal discharge, the previous paper (1967) suggests that there is also an element of reflex inhibition of sympathetic tone to the heart in the diving response. With respect to the heart ventricles a sympathetic inhibition in general implies a reduction of contractile force other factors being equal. However such a mechanism can hardly be the only or even

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major background of the reduced ventricular force during submersion since it was observed that also after complete β-blockade of the heart, stroke volume was still considerably reduced.

The present paper deals with the question whether the negative inotropic influence during diving, which evidently can only in part be due to a reflex reduction of the sympathetic tone may be due to a direct vagal influence on the ventricles. The results of this study have been briefly reported elsewhere (Folkow *et al.* 1966)

Methods

Ducks, weighing 2.5—3.0 kg were anesthetized with nembutal (30 mg/kg) and placed in supine position with constant artificial respiration. The left ventricular pressure and the heart rate were measured through a cannula threaded into the ventricular chamber by way of the right brachial artery. In some experiments the central venous pressure was measured by a catheter in the left brachial vein, with its tip placed in or close to the right atrium. The arterial pressure, when measured, was obtained from a cannula in the left carotid artery. All pressures were measured with Statham P 23A or B pressure transducers and recorded on a Grass Model 5 polygraph.

Cardiac output was determined by the thermodilution method (Fegler 1954, Korner 1965). A thermistor was placed in the aortic root via the left brachial artery. A venous cannula was inserted through the right brachial vein with its tip close to the right atrium and connected to a reservoir arranged for injecting a slug (0.3—1.0 ml) of saline at room temperature.

The vagal nerves were isolated in the mid-cervical region, sectioned and their peripheral ends were placed in chloridized silver ring-electrodes connected to a Grass S-4 stimulator. Pacing the ventricles was accomplished by one of two methods: 1. A large needle (N 15) was pushed through the thick breast muscle and the thin portion of the sternum until the heart was touched but not punctured by the needle tip. A bipolar electrode, insulated except at its tip, was then passed through the needle and pushed into the ventricular muscle tissue. Subsequent examination revealed that the tips of the electrode were placed in the wall of the left ventricle. 2. A more satisfactory method was to drill a hole on either side of the sternal crest just over the heart. Through a small slit in the pericardium two thin electrode plates (10 × 15 mm) were inserted on either side of the ventricle. They were held in close contact with the ventricular sides by means of bone wax pressed into the opening in the sternum. A second Grass stimulator was used to pace the heart at a rate slightly above the resting rate.

The arterial oxygen content was measured with a F & M blood gas chromatograph.

Results

Non-paced heart

The classical response of the mammalian heart *in situ* to stimulation of the efferent vagal nerve fibres at submaximal frequencies is a decreased rate, an increased end-diastolic ventricular pressure and an increased ventricular peak-systolic pressure, here illustrated in the cat (Fig. 1 A). The increased filling time, secondary to the reduced heart rate, leads to an increased end-diastolic volume of the ventricle and, hence, by the Frank-Starling concept, an increased strength of contraction ensues.

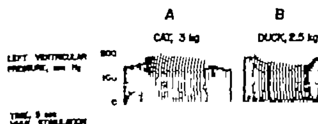


Fig. 1. A. Left ventricular pressure measured in a cat during low frequency vagal stimulation. B. Left ventricular pressure measured in a duck during low-frequency vagal stimulation.

When the vagal fibres of the duck were stimulated at submaximal rates, the heart rate slowed and the end-diastolic pressure increased as in the cat. Nevertheless, the ventricular peak-systolic pressure practically always decreased (Fig 1 B) except occasionally when there could occur an increased pressure for the first few beats.

Paced heart

If the heart of the duck was paced so as to eliminate the negative chronotropic effect of the vagal stimulation, a clear reduction of the ventricular peak-systolic pressure ensued despite the fact that the end-diastolic pressure increased. The extent of this vagal influence could be quite marked, often reducing the peak-systolic pressure to less than half the control value at frequencies that must be considered to be within the physiological range (5—10 imp) as is shown in Fig 2. The by far greatest part of the decrease in ventricular peak-systolic pressure during the vagal stimulation occurred within 5—10 seconds after stimulation was started. When the vagal stimulation was stopped, the ventricular peak-systolic pressure and the end-diastolic pressure returned to the control value within 5—30 seconds.

These events are also clear from Fig 3 where intermittently the catheter in the left ventricle was slightly withdrawn so as to record the aortic pressure instead. During the control period mean arterial pressure was 20—50 mm Hg below ventricular peak-systolic pressure, but only 10—20 mm Hg below this pressure during vagal stimulation, as is clear from B in Fig 3 If the pacing of the ventricle was suddenly stopped during a period of vagal stimulation, both peak-systolic pressure and end diastolic pressure increased, though not to the control level, and the heart rate decreased (Fig 4)

Cardiac output, measured by the thermodilution method, decreased during vagal stimulation even when the heart was paced at a constant rate, implying that the stroke volume decreased proportionately to the reduced output (Fig 5) A curve relating effector response to frequency of vagal stimulation obtained from one experiment by stimulating the vagi with supramaximal strength but with stepwise increases of frequency between 2 to 20 stimuli/sec, is shown in Fig 6 The curves indicate that most of the response to vagal stimulation took place at frequencies below 5/sec, as is characteristic for most autonomic neuro-effectors (*cf* Folkow 1955)

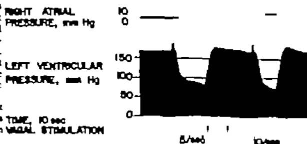


Fig. 2. The heart of the duck was paced at constant rate Recordings of right atrial pressure and left ventricular pressure The vagal nerves were stimulated with supramaximal strength at 5 and 10 imp. Note the profound reduction in left ventricular systolic pressure and the rise in left ventricular diastolic pressure and in right atrial pressure The background of the initial slight increase in end-systolic pressure is not known, but this phenomenon was occasionally observed.



Fig. 3 The heart of the duck was paced at constant rat
A. The effect of graded stimulation of the agal nerves on central venous pressure and left entricular pressure.
B. The effect of stimulation of the vagal nerves on left ventricula pressure and aortic pressure.



Fig. 4 The effect of stopping the pacing of duck heart during period of constant vagal stimulation t submaximal strength and rate

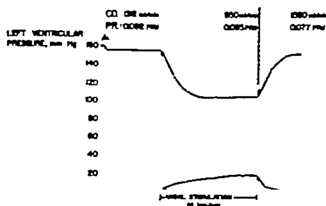


Fig. 5. The heart of the duck was paced 340 beats/min. The effect of vagal stimulation on left ventricular pressure, cardiac output and total resistance.

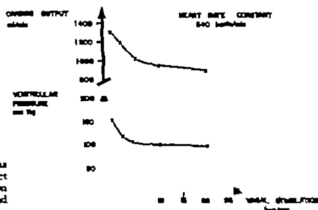


Fig. 6. The heart of the duck was paced at 540 beats/min. The effect of frequency of vagal stimulation on left ventricular pressure and cardiac output.

One possible cause of the decreased peak-systolic pressure and cardiac output during vagal stimulation while the heart was paced might be a gross decrease in peripheral vascular tone, resulting in a decreased effective circulating blood volume Thus would decrease the venous return and thereby reduce cardiac output. However if this were true and if the ventricular function were not decreased by the vagal stimulation, then the end-diastolic pressure would not be expected to rise as it did. To ascertain whether vagal stimulation really has any decreasing effect on the peripheral resistance, the peripheral resistance was calculated as the arterial pressure — venous pressure/cardiac output before, during and after stimulation of the vagi (Fig 5) The data indicate that the resistance was essentially unchanged. Therefore the decreased ventricular pressure during vagal stimulation must result from a change of the contractile strength of the ventricular myocardium. Further proof of this was obtained by cutting the vagi below the level of the heart and showing that the decreased ventricular pressure still occurred during vagal stimulation.

An additional test, establishing that the decreased peak-systolic pressure during vagal stimulation was due to decreased ventricular function, was obtained by infusing blood intra-arterially during vagal stimulation at such a rate as to maintain the control arterial blood pressure largely unchanged. If ventricular contractility is reduced, then the end-diastolic pressure should increase. Fig 7 shows that, indeed, the end-diastolic pressure was increased.

The decrease in peak-systolic ventricular pressure caused by vagal stimulation may possibly be a result of an anoxic state of the ventricular muscle produced either directly or indirectly by the vagal stimulation. The mechanisms which might be postulated to cause anoxia are coronary vasoconstrictor fibres in the vagal nerves, decreased arterial pressure reducing coronary flow or vagal fibres producing bronchiolar constriction with reduced alveolar ventilation thereby reducing the oxygen supply to the myocardium. Therefore, experiments were performed to exclude these possibilities.

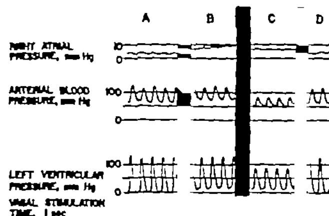


Fig 7 The heart of the duck was paced at 360 beats/min. The effect of intra-aortic blood infusion on right atrial pressure arterial blood pressure and left ventricular pressure during vagal stimulation. A and D control periods. During B and C the vagal nerves were continuously stimulated at 8 impulses per second, but in B arterial pressure was kept largely the same as in the control periods by constant infusion of blood into the aorta. Note the considerable rise in left ventricular end-diastolic pressure during this procedure. The rise is also evident in C, even though the aortic blood infusion had been stopped.

Samples of arterial blood, withdrawn before and during the period of vagal stimulation were analyzed for oxygen content and showed no decrease. If anything a slight increase in oxygen content occurred. Peak-systolic pressure was, nevertheless, markedly decreased during the vagal stimulation, as was cardiac output. In another experiment the trachea was clamped for approximately 60 seconds with no alteration in the peak-systolic pressure of the ventricles. These data prove that the negative inotropic effect of vagal stimulation is not indirectly caused by any reduction in arterial oxygen content It is true that if the vagal stimulation was continued for a longer time (70 sec) a slight decrease in the arterial oxygen saturation of the arterial blood could occur but this occurred long after the reduction in cardiac output and stroke volume was already fully established. Thus, this retarded slight reduction in blood oxygen content seemed to be a hemodynamic consequence of the vagal depressing effect on the heart rather than its cause and when it appeared arterial blood pressure had often fallen to some 40 per cent of the control value as a result of the depression of the heart activity

To exclude the remote possibility that the negative inotropic effect of vagal stimulation was indirectly produced by myocardial hypoxia, as a result of activation of vagal coronary vasoconstrictor fibres, the following experiments were performed. Blood flow through the cross-perfused coronary vascular bed was measured with an optical drop counter connecting the donor duck with the arterial inflow to the coronary vessels of the recipient duck Acetylcholine, injected into the coronary vessels of the cross- perfused duck heart, invariably produced coronary vasodilatation Therefore cholinergic vasoconstrictor fibres would not appear to be present. Moreover stimulation of the vagal nerves to the heart of the recipient duck had no effect on its coronary blood flow Furthermore any adrenergic coronary vasoconstrictor fibres that might exist in the vagal nerves to the duck's heart cannot be responsible for the vagally induced negative inotropic effect on the ventricles. Such a hypothetical adrenergic mechanism could also be excluded by the fact that the

negative inotropic effect of vagal stimulation was just as efficient after the duck had been given guanethidine in addition to both α- and β-blocking drugs (Regitin® and Häsle 26/58, respectively) in amounts that are generally recognized to produce a complete block of adrenergi nerve influences.

Evidently the vagal negative inotropic influence on the duck's ventricles must be mediated by a direct effect of vagal fibres on the ventricular myocardium. This direct parasympathetic effect could be completely blocked by atropine (0 1—0.2 mg/kg) and it is powerful enough to reduce stroke volume to about half the control value, other parameters being kept constant.

Discussion

A possible negative effect of vagal stimulation on the ventricle of most conventional laboratory animals is masked by the positive inotropic effect of increased filling of the ventricle due to a negative chronotropic effect (Frank-Starling concept) The negative inotropic effect of vagal stimulation has been contested or suggested by many workers. These have been adequately reviewed recently by De Geest *et al.* (1965) who have also demonstrated conclusively that vagal stimulation to the paced, isovolumetric left ventricle of the dog caused a reduction of the ventricular systolic pressure. In other experiments they have controlled the peripheral resistance and venous return with th same results, that vagal stimulation produces a negative inotropic effect on the ventricle.

Contrary to the conventional laboratory animal, the duck ventricle shows a decreased peak-systolic pressure and increased end-diastolic pressure when the vagi are stimulated. It appears that either the Frank Starling concept does not apply to the duck's heart — which is *a priori* very unlikely — or that the negative inotropic effect of the vagi on the ventricle has a very marked effect. The experiments with paced hearts show that the vagal negati e inotropic effect on the ventricle is very marked, indeed. In the experiment (Fig 4) in which the pacing of the ventricle was suddenly stopped during the vagal stimulation, the heart rate slowed and the peak systolic pressure increased, though not to the control level. This strongly indicates that the duck ventricle does adhere to the Frank-Starling concept. Also, occasionally vagal stimulation of the unpaced ventricle will cause an increased peak-systolic pressure for a few beats before t decreases. Therefore the response of the ventricle to reflex vagal stimulation in intact, non-anesthetized ducks will be a balance between the Frank-Starling forces and the pronounced negative inotropic effect on the entricle w th probable dominance of the negative inotropic effect.

The data of these experiments and the previous paper (1967) on the diving re sponse clearly indicate that the reflex vagal inhibition of the heart entails not only a negati e chronotropic effect but also a negati inotropic effect on the entricles. This effect combined with th reflexly reduced positive chronotropic effect of the sympathetic fibres, is pronounced enough to reduce the stroke volume during s bmersion despit often profound rise n central venous pressure (Folkow Nils-

son and Yonce 1967) These findings are consistent with the duck's mechanisms to conserve oxygen during a dive and might be relevant for most, or all divers. The work of the heart during the dive will not only be a function of the decreased heart rate but will also be dependent on the strength of the beat. With the reflex negative chronotropic and inotropic effect of vagal stimulation during the dive, the oxygen demand of the myocardium should decrease, allowing maximum utilization of the oxygen reserve by the central nervous system.

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About Arterio-venous Shunts in Salivary Glands

A Study with Krypton Elimination Technique in Dogs

By

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Abstract

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In 5 animals the krypton (Kr^{85}) elimination rate from the exposed submaxillary gland was studied. Both local and intra-arterial administration of the indicator isotope was used before, during and after chorda stimulation. The results demonstrate a tenfold increase in tissue blood flow during chorda stimulation and strongly suggest that this increased flow passes through capillaries, with no signs of any opening of arterio-venous shunts.

The existence of arterio-venous shunts in the salivary glands has been discussed for a long time Histological evidence of such shunts is given for the salivary glands (Holzlöhner and Niessing 1936, Spanner 1937 1942) as well as for most other organs (*cf* Clara 1956)

It has, for example, been assumed that the opening up of these anastomoses during chorda stimulation should be responsible for the striking increase of blood flow (*cf* Burgen and Emmelin 1961) and it has even been proposed that capillary flow in the secretory alveoli decreases during the stimulation (Holzlöhner and Niessing 1936)

By use of the freely diffusible gas technique (Xe^{133} or Kr^{85}) it is possible to evaluate blood flow in different organs and tissues. After intra-arterial administration the clearance curve will reflect both nutritional blood flow and flow through arterio-venous shunts (Häggendal *et al* 1965) After local microinjections of the tracer its elimination will reflect only the nutritional flow In case the clearance curves for the two administration routes exactly coincide this seems to exclude the presence of a shunt flow

The present study was undertaken in order to evaluate the type and magnitude of the blood flow increase occurring in the salivary glands upon chorda stimulation and to explore whether part of this flow increase takes place through arterio-venous shunts

Methods

The study was performed in 5 mongrel dogs during light pentobarbital anaesthesia.

The submaxillary glands were exposed uni- or bilaterally. The internal carotid artery was ligated and all branches from the external one were also ligated with exception for the artery that supplies the submaxillary gland. I proximal small artery thin polyethylene catheter was inserted. Th horda tympani was dissected free and placed on a bipolar stimulating electrode. The stimulation parameters were 3—5 v 2 msec and 20 imp/sec: this frequency being known to produce a definit ly maximal response. The salivary flow was observed at the orifice of the salivary duct.

Changes in blood flow in th salivary gland were evaluated from the changes in elimination rate of administrated radioactive krypton-saline solution (Kr^{85}). The intra-arterially injected krypton wa given in amounts of 1—2 ml into the external carotid rtery. The local krypton injection amounted to 1—5 µl and were given by means of a small syringe (no. 701 Hamilton Ltd Whitbier California) with a thin needle (0 4 mm external diameter)

As rule the γ-activity was registered after intra-arterial i jections and the β-activity after local injections

Changes in blood flow are expressed as differences in the half-time value of the krypton activity elimination rate. Absolute figures of the blood flow are thus not given due to the fact that the partition coefficient of krypton between the gland tissue and blood was not determined.

Results

The experiments in all the five animals were conclusive A typical example is shown in Fig 1

Prior to stimulation of the chorda tympani when no salivary secretion could be observed the krypton elimination, curves, both after intra-arterial and local injections, were fairly mono-exponential in character with a half-time of about 3 min

After previous *local* administration of the isotope there was an almost instantaneous increase of the krypton elimination rate when a maximal chorda activation was started with an about tenfold decrease of the elimination half time the curve being still mono-exponential looking After *intra-arterial* Kr^{85} injections chorda stimulation produced the same pronounced increase in the elimination rate of the tracer When semilogarithmically plotted these curves proved to be composite and when dissol ed in two components the fast one had a half-time almost identical to the half-time of the local injection curve during stimulation. The half time of the slow component, however was of the same order of magnitude as that of the curves recorded during rest.

Discussion

The experiments demonstrate that the blood flow of the salivary glands increases about tenfold during maximal stimulation of the cholinergic nerve supply to the gland and this increase of flow must pass through capillaries. The composite curves after intra-arterial injections during stimulation show however that some parts of the glands do not increase th flow compared to the resting values. The significance of these slow components are hard to evaluate, but it is most likely that connective tissue of the glands, inj ries of the nerves during preparation causing ineffective

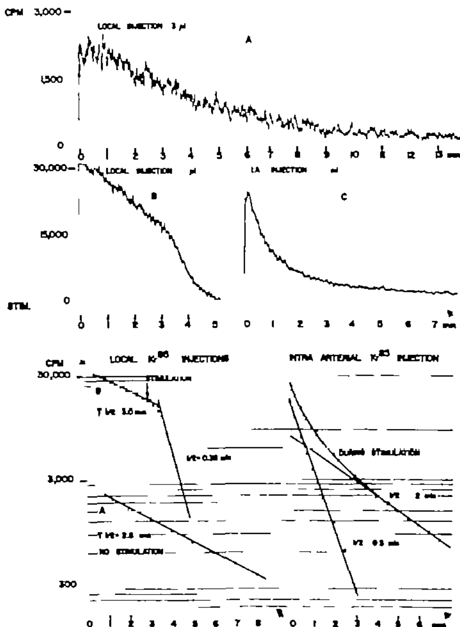


Fig. 1. Above: Kr^{85} elimination curves from one typical experiment. Wash out curves after local and intra-arterial Kr^{85} administration to the submaxillary gland. A. Local injection before nerve stimulation. B. Local injection. After some minutes stimulation of the cholinergic nerve to the gland is started. The stimulation parameters are 5 V, 2 msec and 20 imp/sec. C. Intra-arterial injection during nerve stimulation. *Below*: Semilogarithmic plot of the same curves. Curve C is resolved into two components.

stimulation to parts of the glands are factors which may be involved. A tenfold increase of capillary blood flow through the gland is in good agreement with the flow increases seem during direct blood flow measurements and maximal chorda stimulation (Terroux *et al.* 1959) but with such direct recordings it cannot be determined whether the flow in part passes through opened arterio-venous shunts. The identical rapid phases of the Kr^{85} elimination curves after intra-arterial and local administration that were seen in the present experiments upon chorda stimulation speak against any significant involvement of shunt flow. Furthermore there was not initial "peak" in the elimination curves after intra-arterial injections of Kr^{85} as was the case when patients with traumatic arterio-venous shunts were studied with this technique (Häggendal *et al.* 1965).

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Acid Base Relations in Arterial Blood and Cerebrospinal Fluid of the Unanesthetized Rat

By

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Abstract

PONTÉN U and B. K. SIESJÖ. *Acid-base elations in arterial blood and cereb o-spinal fluid of the unanesthetized rat* Acta physiol. scand 1967 *71* 89—95

A technique is described for collection of arterial blood in unanesthetized rats and for removal of cisternal cerebrospinal fluid (CSF) after anesthesia in such way that the acid-base parameters can be considered to represent the unanesthetized state. I uninjected rats the plasma pH was found to be 7 47 the pCO_2 38 5 mm Hg and the plasma bicarbonate concentration 7.3 meq l When such rats were made acidotic or alkalotic by means of intraperitoneal injections of acid nd basic solutions both the CSF bicarbonate concentration, and the CO_2 tension, varied linearly with the plasma bicarbonate concentration, but the changes in the CSF bicarbona concentration were only 35 per cent of the corresponding plasma hanges. There were small but significant changes (0 04 pH units) of the CSF pH in the same direction as in blood and there was thus no indication of paradoxical shifts in the CSF pH. The implication of these findings for current theories regarding the regulation of respiration is discussed.

Studies of chronic acid-base disorders n humans have shown that the cerebrospinal fluid (CSF) pH is regulated within narrow limits in spite of wide variations in the acid-base parameters of the blood (for references, see Mitchell *et al* 1965) The mechanisms underlying this regulation ha e not been precisely defined although it has been proposed that active transport of hydrogen or bicarbonate ions is involved (Pappenheimer *et al* 1965 Fencl *et al.* 1966 Severinghaus 1965) The further study of these mechanisms requires experiments on animals and preferably on unanesthetized animals. Such experiments ha been described in the goat (Fencl *et al* 1966) and in the rat (Siesjö and Pontén 1966) but the technique used in the latter study necessitated that the nimals used were anesthetized prior to the sampling of blood and CSF In the present paper a technique is described for sampling rterial blood from the unanesthetized rat and for obtaining CSF in such a way that the acid-base alues could be assumed to be representative of the unanesthetized rat When the animals were subjected to nonrespiratory acidosis and alkalosis for a period of 6 hrs. the CSF pH aried directly w th the arterial bi-

carbonate concentration, and there was no paradoxical shift in the CSF pH. The results were thus in close agreement with recent studies on unanesthetized goats by Fencl *et al.* (1966).

Methods

The experiments were performed on male rats of the Wistar strain, weighing 250—400 g. The middle caudal artery was cannulated in light ether anesthesia. The artery was exposed by a ventral incision about 2 cm from the base of the tail. The inferior caudal nerve trunk was excised. The wound was then closed and covered tightly with circular turns of an adhesive tape. The rat was left to recover from the anesthesia in a perspex box with the arterial cannula drawn out through a hole in the bottom of the box. The cannula was flushed with minute amounts of dilute (40 mg/kg) heparin solution at intervals of 20—40 min. The flushing of heparin and the sampling of blood did not cause any noticeable reaction of the animal. The technique permitted repeated arterial samples to be withdrawn during several hours. At the end of the experiment, and after at least two sets of measurements of the blood acid-base parameters had been made, the animal was anesthetized with intraarterial Pentobarbital (Nembutal) in a dose of 60 mg/kg. As soon as surgical anesthesia was attained the animal was quickly removed from the box, the head was fixed in a headholder and CSF was withdrawn from the cisterna after previous exposure of the atlantooccipital membrane. The CSF samples were collected in glass capillaries. The blunt end of the capillary was attached via a plastic tubing to a mouthpiece permitting the collection of 50—80 μl by gentle suction. With some practice the lag between the injection of Pentobarbital and the collection of the CSF could be reduced to below 3 min.

The pH, the pCO_2, and the hemoglobin concentration of blood, the chloride concentration of plasma, as well as the total CO_2 content (T_{CO_2}) and the chloride concentration of CSF were measured. The remaining relevant parameters in blood and in CSF were then derived as previously described (Siesjö 1962, Pontén and Siesjö 1966, Pontén 1966, Siesjö and Pontén 1966). The capillary bicarbonate concentration was calculated to facilitate derivation of CSF/plasma ratios (Potén 1966). The pH of the CSF was calculated from the bicarbonate concentration and the CO_2-tension of the CSF using a pK' of carbonic acid of 6.125. The bicarbonate concentration was calculated from the T_{CO_2} by subtracting the amount of CO_2 dissolved, using the solubility coefficient 0.0314 mmoles/l mm Hg (Siesjö 1962), while the CSF CO_2-tension was derived from the arterial CO_2 tension measured immediately before the induction of anesthesia (Pontén and Siesjö 1966). The pH and the pCO_2 values were measured at 37.5° C.

There were two types of experiments. In the first type only arterial blood was sampled and repeated samples were collected for periods of up to 5 hrs in order to establish only variation with time in the acid-base parameters. In the second type of experiments 3 groups of rats received 3 intraperitoneal doses of either a simulated extracellular fluid, an isotonic $NaHCO_3$ solution, or an isotonic $NH_4Cl/NaCl$ solution. The injections were given in amounts of 3 ml per 100 g body weight every 2 hrs (for details of techniques, see Siesjö and Pontén 1966). At the end of the equilibration period (6 hrs) the animals were anesthetized with ether and the arterial cannula was inserted. About 20 and about 30 min, respectively, after the animal had recovered from the anesthesia and had begun to walk around in the box arterial samples were drawn, whereafter Pentobarbital was injected and CSF was withdrawn as described above.

Results

Control experiments. Apart from the main experimental groups a control group of 16 animals was made. In all these control rats, as well as in 6 of the animals belonging to the main experimental groups the body temperature was measured with a thermocouple electrothermometer either just before or a few min after the withdrawal of CSF. In those 22 experiments the mean temperature was $37.7° C \pm 0.2$ (mean ± s.e.). Since this temperature did not differ appreciably from the temperature of the pH and the pCO_2 electrodes and since adequate protection of the thermometer lead involved a restriction of the movements of the animals, no further temperature measurements were made.

In 6 animals the arterial CO_2-tension was measured before the injection of barbiturate and after the ttainment of surgical anesthesia The mean CO_2-tensions were 37 8 and 42.5 mm Hg, respectively This means that the injection of Pentobarbital led to a mean increase in the arterial pCO of 4 7 mm Hg during the 2—3 min required to sample the CSF

In 6 other experiments CSF was sampled for measurement of the CO_2-tension. In this group the mean arterial CO_2-tension before anesthesia was 40 1 mm Hg This tension corresponds to a CSF CO_2-tension of 46.4 mm Hg (Pontén and Siesjö 1966) The mean CSF CO_2-tension measured after anesthesia was 50.2 mm Hg, implying that the increase in the CSF CO_2-tension due to the short anesthesia was 3.8 mm Hg The result was thus in good agreement with that predicted from the measurements on arterial blood

I I riations i the acid-base parameter of arterial blood with time If the first arterial sample was drawn 20 min after the animal had recovered from the the ether anesthesia there were no systematic changes in any of the acid-base parameters of arterial blood during the periods studied, although the scatter of the individual values was larger during the first 2 hrs (Fig 1) Since there were no systematic variations with time in the pH the pCO_2 or the HCO_3^--values, mean values were calculated for each individual rat, and the means for the 6 rats were then tabulated (Table I uninjected group) It is seen that the CO_2-tension was close to 40 mm Hg, and that the actual bicarbonate concentration was 27.3 mEq l, corresponding to the high pH alue of 7 468.

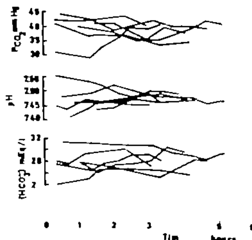


Fig 1 pCO_2, pH and standard bicarbonate alues for rterial plasma of six unanesthetized rats, followed for periods up to 5 hrs after recovery from ether anesthesia The standard bicarbonate alues were calculated from the pH, the pCO_2 and the hemoglobin alues of whole blood, using tandard homogram

II R lation between the arterial plasm and th CSF acid-base pa amete In order to obtain a control group to animals made acidotic or lkalotic 6 rats were given injections of a simulated extracellular fluid (see Siesjö and Pontén 1966) None of the acid-base alues obtained in the control group (Table I blank group)

TABLE I Arterial plasma and CSF acid-base parameters in unanesthetized rats. I the ... had ecovered from ether nesthesia. I six of these animals repeated samples were ... values from 15 other rats i yield th mean values in the table. The remaining three ... with a NH Cl-N Cl solution, or with N HCO$_3$ solution for period of 6 hrs befor ... the table refer to samples drawn 20—30 min after the animals had recovered from the

Arterial plasma

Type of exp.	pH	Pco mm Hg	Hemogl. concentr mg/100 ml	Act.HCO- meq/l	Stand. HCO- meq/l
Uninjected	7 468	38.5	14 1	27.3	27 7
(21)	0 006	±0.5	±0.3	±0.5	±0.4
"Blank"	7 499	38.1	15.2	28.9	29.2
(6)	±0.011	±1.5	±0.4	±1.2	±1.0
NH Cl	7 339	34 9	15.6	18.5	19.4
(9)	0 014	±0 7	±0.7	±0 5	±0.5
NaHCO	7 565	42 7	15 5	38.5	37.9
(8)	±0.009	±1 1	±0.7	±0.7	±0.6

differed significantly from the values in the uninjected group of animals, indicating that the procedure followed in the two main types of experiments (see methods) were comparable. Since the main interest in the second type of experiments concerned th acid-base changes obtained in acidosis and alkalosis, respectively only differences obtained between these two groups will be discussed. There was a highly significant difference between the pH the pCO_2, the actual HCO_3^- the standard HCO_3^- and the chloride concentrations in blood between the acidotic and the alkalotic groups ($p<0.001$). There was also a highly significant difference in the HCO and the chloride concentrations in the CSF between the same groups ($p<0.001$) and a small but significant difference between the CSF *pH* values ($p<0.05$). It can be seen from the table that the difference in actual plasma bicarbonate between the acidotic and the alkalotic groups (20 meq/l) was accompanied by a reversed and equal change in plasma chloride concentration (19.8 meq/l). The bicarbonate change in CSF was only 32 per cent (6.4 meq/l) of the change in arterial plasma but also this change was accompanied by a corresponding change in the chloride concentration (6.6 meq/l). It can also be seen that the pH difference between the acidotic and the alkalotic groups wa 0.23 units in arterial blood as compared to 0.04 units in the CSF.

Discussion

A. Experimental t chniq The present technique for obtaining arterial blood has the advantage that the acid-base parameters are not influenced by any anesthetic. There are also good reasons to believe that the derived CSF acid-base parameters are influenced to a very slight degree by the anesthesia induced prior to sampling of CSF. This is supported by the following facts. The pH of the CSF is given by the

jected group plasma and CSF were sampled after minimum period of 20 min after the animal drawn for periods of up to 5 hrs (see Fig 1) Mean values for these animals were pooled with groups of the table refer to animals injected i.p. with simulated extracellular fluid (blank") they were anesthetized with ether for the cannulation of the artery The acid-base values given in anesthesia.

	CSF					
Capill. HCO⁻ meq/kg of H_2O	Cl⁻ meq/l	Tco mmoles/l	Pco mm Hg	Act.HCO⁻ meq/l	Cl⁻ meq/l	pH
33.0	103.6	29 0	45.0	27.6	127 1	7 417
±1.2	±1.6	±0.9	±0 6	±0.9	±1 1	±0.007
22.0	113.7	25 4	41.8	24.1	129.4	7.388
±0.3	±1.2	±0.6	±0.6	±0.5	±1.3	±0.008
43.0	93.9	32.1	48.9	30.5	122.8	7 426
±0.8	±1 4	±0.5	±1 1	±0.5	±1 4	±0.009

CO_2-tension and the bicarbonate concentration. The CSF CO_2-tension can be accurately calculated from the arterial CO_2-tension (Pontén and Siesjö 1966) which is measured in the unanesthetized state. The total CO_2-content can be measured in CSF withdrawn within 3 min after the intraarterial injection of the barbiturate Since the CSF is virtually unbuffered against CO the bicarbonate concentration can only change significantly by means of passive or active fluxes between the CSF and the blood, or between the CSF and the tissue proper It can be safely assumed that there will be no such significant fluxes within this short time (cf Pontén 1966) This implies that the only possible change in the CSF is an increase in the total CO_2-content due to a transient increase in the CSF CO_2-tension. Thus, if the CO_2-tension is increased by e.g. 5 mm Hg during the anesthesia there will be change in the total CO_2-content of the CSF of about 0 15 mEq l. Control experiments with withdrawal of arterial blood or CSF 2—3 min after the induction of the anesthesia indicate that any increase in the CO_2-tension due to the anesthesia did not exceed 5 mm Hg This corresponds to an error in the calculated CSF bicarbonate concentration of less than 1 per cent due to the anesthesia.

A comparison between the present arterial values and those obtained in Pentobarbital anesthesia (Pontén 1966, Siesjö and Pontén 1966) reveals that the anesthesia is followed by a 2—3 meq l decrease in the actual and standard bicarbonate concentrations and, since the arterial CO_2-tension seems to be very little influenced by the amount of anesthetic used, by corresponding reduction of arterial pH The CSF acid-base parameters obtained in the present material of unanesthetized rats are very similar to the corresponding values obtained on anesthetized rats as reported in a previous communication (Siesjö and Pontén 1966 There appears to be a slightly higher CSF bicarbonate concentration in the previous

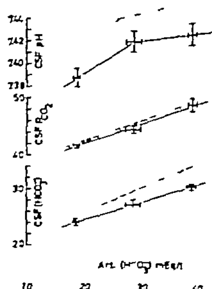


Fig. The actual bicarbonate concentration, the pCO_2, and the pH values of CSF in unanesthetized rats related to the bicarbonate concentration of arterial plasma (means ± e.) The animals were injected i.p. with simulated extracellular fluid, with an isotonic $NaHCO_3$ or with an isotonic NH_4Cl-NaCl solution for 6 hrs prior to sampling of blood and CSF The diagram was chosen to facilitate comparison with previous results on rats anaesthetized before sampling of blood and CSF (brooken lines, Siesjö and Pontén 1966) and with recent results on unanesthetized goats (Fencl *et al.* 1966)

groups. but the change is not significant. In order to facilitate comparisons between these two groups and previously reported values for unanesthetized goats, the values were plotted in an identical diagram to that described by Fencl *et al* (1966 Fig 2

The arterial acid-base parameters measured in the present groups of unanesthetized rats are in disagreement with values reported by Simmons, Kahn and Gurz (1966 *These authors report an arterial pH of 7 48 a CO_2-tension of 2 mm Hg* and a base excess of — 1 meq l. This implies that the standard bicarbonate was about 23 meq l. Thus, although the actual pH was comparable to that measured in the present experiments, the CO_2-tension was more than 10 mm Hg lower and the standard bicarbonate more than 5 meq l lower than in our own groups. The technique used by the authors of puncturing the left ventricle of the heart may have influenced the acid-base parameters to a significant degree.

B. Implication of the CSF changes In a previous communication (Siesjö and Pontén 1966 it was reported that when an identical procedure was used for producing acidosis and alkalosis in unanesthetized rats which were subsequently anesthetized before sampling of blood and CSF a plasma pH change of 0.2 units was accompanied by CSF pH change of 0 05 units. In the present series a plasma pH change of 0.23 units between the acidotic and the alkalotic groups was accompanied by a CSF pH change of 0 04 units. Although these results confirm previous reports that there are very small changes in the CSF pH in nonrespiratory acidosis and alkalosis, they are in agreement with the recent results of Fencl *et al.* (1966) in showing small but significant changes in the CSF pH in the same direction as those in arterial blood. These results have important implications for the theories regard-

ing the regulation of respiration. Thus, on the basis of the results obtained in humans and in experimental animals it has been assumed that a nonrespiratory change in the acid-base status of the blood primarily leads to a reverse ("paradoxical") shift in the CSF pH due to the effect of arterial (H) on the peripheral respiratory chemoreceptors (Severinghaus 1965 Mitchell *et al.* 1965) In other words, a nonrespiratory acidosis with accumulation of hydrogen ions in the blood would be accompanied by hyperventilation which by reducing pCO_2 gives rise to an alkaline CSF pH. It is then assumed that the CSF pH is reduced to normal d e to active transport of hydrogen or bicarbonate ions between blood and CSF In view of the results of Fencl *et al.* (1966) and of the present results this hypothesis appears less probable. Thus, the hypothesis would require that there exists an active transport mechanism which regulates an alkaline pH beyond the normal point to an acid pH or the reversed way in nonrespiratory alkalosis. A more probable hypothesis is that in a metabolic acidosis hydrogen ions diffuse into the extracellular compartments (ECF) of the brain, invoking similar cid-base changes in the blood and the ECF The regulation of CSF pH to near normal alues would then be the result of (1) the influence of the increased ECF acidity upon central respiratory chemoreceptors, with resulting hyperventilation, of (2) an increased electrical potential between CSF nd blood (which tends to draw bicarbonate ions into the CSF compartments) and, possibly of (3) active transport mechanisms (cf Fencl *et al.* 1966 Goodrich 1965)

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Protection of the Gastric Mucosa against the Lesions Caused by Reserpine through Degranulation of Mucosal Mast Cells

By

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Abstract

Räsänen, T. and E. Taskinen. *Protection of the gastric mucosa against the lesions caused by reserpine through degranulation of mucosal mast cells.* Acta physiol. scand. 1967. 71. 96—104.

The ulcerating effects of reserpinization in the gastric mucosa of rats were studied by special observation of the function of the gastric mucosal mast cells and the changes in mitotic activity of the epithelial cells. The significance of degranulation of the mucosal mast cells for the ulcerating effect of reserpine was also studied after the pretreatment of rats with dexamethasone. Immediately after the dexamethasone treatment there was a pronounced decrease in mitotic count of the gastric mucosal epithelium which 2—3 days later when the mast cells of the gastric mucosa were almost completely degranulated was followed by a 2—3 fold increase in mitotic activity as compared with controls. 10 and 20 days later the mitotic count in the gastric mucosal epithelium decreased below the controls values, the mucosal mast cells being re-granulated over the control values. The changes in mitotic count in the epithelium of duodenum, ileum and colon under the same conditions were scanty. The degranulation of the mast cells was markedly slighter in the intestinal than in the gastric mucosa. In the reserpinized rats the number of mitoses in the gastric epithelium decreased simultaneously with the degranulation of the mast cells. The degranulation of the mast cells in the duodenal mucosa was slighter and no changes in mitotic activity occurred in the duodenal epithelium. Lesions appeared during reserpinization in the ventricular mucosa. Reserpinization after the dexamethasone treatment did not cause ulcer formation in the gastric mucosa, nor did it in this phase cause inhibition in the mitotic activity, typical of which was an overshooting 2—3 days after dexamethasone treatment. The mechanism of gastric mucosal ulceration is discussed.

Hemorrhages and epithelial lesions occur in the gastric mucosa of rats and mice in the course of degranulation of the mucosal mast cells during stress on the action of ACTH, glucocorticoids and especially of insulin. When degranulation of mast cells has taken place no lesions occur in the gastric mucosa (Räsänen 1963). Somatotropine which increased the count and the granulation of gastric mucosal mast cells (Räsänen 1960), will to some extent inhibit ulcerogenic effect of the glucocorticoids (Räsänen 1963, Robert, Phillips and Nezamis 1966). In the pylorus ligated ventricles of the hypophysectomized rats the secretion of HCl and hexosamines will return to normal values under the influence of somatotropine (Robert *et al.* 1966).

Reserpine causes a rapid degranulation of the gastric mucosal mast cells in rats (Räsänen and Taskinen 1966) with simultaneous stimulation of the acid secretion (Emås 1963) and results in mucosal lesions (Guerrin *et al.* 1964) In a nonacid isolated pylorus the ulcerogenic effect of reserpine will disappear (Smith and Howes 1964) to return after the exogenic reacidification (Howes *et al.* 1965)

The gastric mucosa will be desensitized against the ulcerogenic effects by prolonged reserpinisation (Clay *et al* 1964) stress (Frenkl, Csalay and Makara 1962) histamine liberation (Jasmin and Bois 1959) and by a long time glucocorticoid treatment (Selye, Jean and Cantin 1960) The blockage of decarboxylation during pyridoxine deficiency will result in prevention of the ulceration of the mucosa in restraint rats (Thayer *et al* 1965)

The exogenic heparin inhibits the epithelial regeneration of the gastric mucosa (Räsänen, Cederberg and Taskinen 1966) and the endogenic heparin in being liberated during the degranulation phase of the mast cells probably acts as a DNA polymerase inhibitor in the gastric mucosa (Lahtiharju, Räsänen and Teir 1964) After complete ACTH induced degranulation of mast cells mitotic activity in the gastric epithelium will increase three-fold (Räsänen and Teir 1961)

The primary factor in the mechanism producing th lesions is probably the vasodilatation and postcapillary spasm caused by 5—HT and histamin which are liberated from mucosal mast cells. These phenomena results in hemorrhages under the superficial epithelium. The osmolarity and the tendency to tissue ruptures are possibly increased in the superficial gastric mucosa following from the rapid fission of the mast granules to the molecular level. The endogenic heparin prevents healing of the lesions by inhibiting the epithelial regeneration and this is possible aggravated in the acid milieu. This mechanism will probably be entirely prevented after th degranulation of the gastric mucosal mast cells.

Method

Male rats of Dawley-Sprague strain aged bout 5 months were used in the study Before initiation of the experiment they were kept 10—14 days in the laboratory for adaptation. During the study the rats were fed standardized mixed diets (Hankkija) and water was given ad libitum.

Immediately after decapitation samples were taken from the gastric and intestinal walls and fixed into fresh 4 basic lead acetat and other samples fixed into Bouin solution were mounted on small piece of cardboard. The specimens fixed into lead acetate were after casting into paraffin, cut ertically to the surface of the mucosa in sections, 10 μ thick. The staining was made in 1 ‰ toluidine blue aqueous solution. The mast cell count of these specimens was performed in visual fields one after another by moving in the direction of the surface. The Leitz microscope with the ide angle plan ocular objectiv system was used the magnification being 500-fold. The diameter of the isual field was 0,36 mm. The samples fixed into Bouin's solution were cut in sections, 4 μ thick, and stained with hemalaun-eosin for determination of the mitotic count. The mast cells and *mitoses were counted in 20 isual fields in the gastric and in 10 visual fields in the intestinal mucosa.*

The study is divided int three different parts

1) Mast cells and the count of the epithelial mitoses in the gastrointestinal mucosa after dexamethasone treatment.

2) Fluctuations in the mitotic activity of the gastrointestinal epithelium nd in the mucosal mast cells during reserpinisation.

3 Effect of eserpine on the gastric mucosa after dexamethasone treatment.

Fisher student t-test was employed for analysis of the statistical significance of the results.

TABLE I Number ± SE mast cells in the gastric body and duodenal mucosa after treatment with dexamethasone of 5 × 1,0 mg

Time after last injection	Number of rats	Mast cells	
		Stomach	Duodenum
4 hrs	10	33 ± 6	56 ± 7
12 hrs	10	17 ± 5	43 ± 5
28 hrs	10	5 ± 1	59 ± 6
54 hrs	10	4 ± 1	43 ± 7
78 hrs	10	7 ± 2	48 ± 8
10 days	10	115 ± 33	179 ± 15
20 days	11	945 ± 48	249 ± 14
Controls	10	593 ± 54	318 ± 17

Results

1 *Mast cells and the count of the epithelial mitoses in the gastrointestinal mucosa after dexamethasone treatment*

The rats were injected dexamethasone (Decadron, Merck Sharp & Dohme), 1 mg × 5, intramuscularly at intervals of 12 hrs. The killing of the rats was performed daily between 12 o clock noon and 2 p.m. except the group killed 12 hrs after the last injection and thus preparated at 8—9 o clock in the morning The controls which were killed together with the 78 hour group were injected with 0,2 ml of saline.

The quantity of the mast cells in the gastric mucosa decreased due to the dexa-

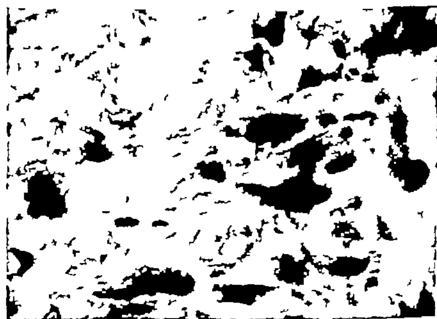


Fig 1 Gastric mucosal mast cells with strong granulation 20 days after dexamethasone treatment Magnification 1200 ×

TABLE II Number ± SE of epithelial mitoses in the gastrointestinal mucosa after treatment with dexamethasone of 5 × 1,0 mg

Time after last injection	Number of rats	Stomach	Duodenum	Ileum	Colon
4 hrs	10	16±4	225±6	215±12	21±4
12 hrs	10	10±3	153±11	146±14	22±5
28 hrs	10	40±6			
54 hrs	10	150±9	302±13	279±27	58±10
78 hrs	10	128±9	293±12	248±20	61±7
10 days	10	36±4	170±11	152±14	42±11
20 days	11	27±3	193±12	167±14	45±6
Controls	10	64±5	197±14	191±16	47±5

methasone treatment and remained low at least 78 hours after termination of the treatment ($P<0{,}001$) (Table I) After 10 days there is reappearance of mast cells and another 10 days later these are more numerous than normal ($P<0{,}001$) (Fig 1) After dexamethasone the number of mast cells will also reduce in the duodenal mucosa but the decrease is not so complete as in the gastric mucosa. Nor will there be any overshooting in the later stage.

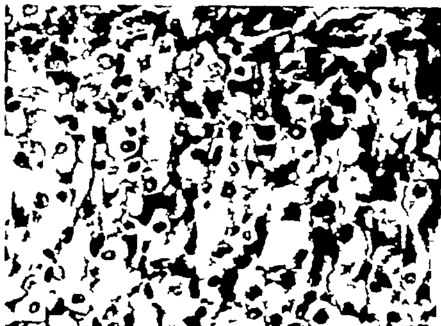
The mitotic count in the gastric mucosa decreased to a quarter ($P<0{,}001$) immediately after termination of the dexamethasone treatment, continuing to decrease during the following 8 hrs (Table II) Twentyfour hours later the number of mitoses begins to increase in the gastric mucosa. After two or three days an abundance of mitoses is encountered in the gastric mucosa the number of which is more than twice as large when compared with the control values ($P<0{,}001$) (Fig 2) This occurs at the time of complete degranulation of the mast cells. 10 and 20 days after termination of the dexamethasone treatment the mitotic count of the gastric epithelium falls below the control values ($P<0{,}001$) during the overshooting time of the mast cells (Fig 3)

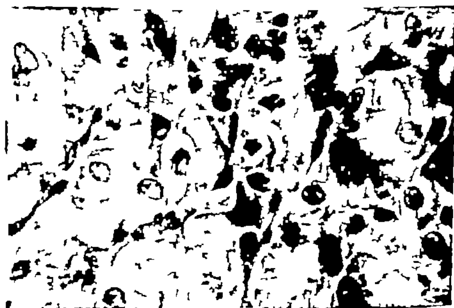
The mitotic variations in the intestinal epithelium to a certain extent follow those in the mucosa of the gastric body while not being equally large. The mitotic overshooting in the duodenal mucosa is readily established ($P<0{,}001$) as well as in the mucosa of the ileum ($P<0{,}02$)

There was not so noticeable a decrease in the mitotic activity of the intestinal epithelium as in the gastric mucosa.

2. *Fluctuations in mitotic activity of the gastrointestinal epithelium and in the mucosal mast cells during reserpinization*

The rats were injected reserpine (Serpasil, Ciba) 2 mg/kg 1 5, and 9 times intra abdominally The reserpine induced degranulation of the mast cells is more pronounced in the gastric than in the duodenal mucosa. These results are published recently (Räsänen and Taskinen 1966) During reserpinization the number of mitoses in the epithelium of the gastric mucosa clearly decreased ($P<0{,}001$) (Table III)





b

Fig. 2. Powerful mitotic activity and cellular dysplasia in the regeneration zone of gastric body mucosa 2 days after the ending of dexamethasone treatment. A. Magnification 400 ×. B. Magnification 1200 ×.

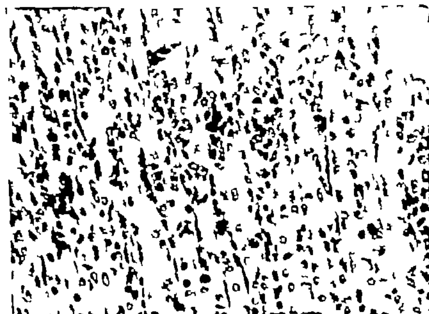


Fig. 3. Reorganization of gastric body mucosa with scanty mitotic activity 20 days after dexamethasone treatment.

TABLE III. Number ± SE of epithelial mitoses and mast cells in the gastric and duodenal mucosa of reserpinized rats

Treatment	Number of rats	Mitoses		Mast cells	
		Stomach	Duodenum	Stomach	Duodenum
Reserpine 1 0,6 mg	8	27±4	209±9	250±17	183±11
Reserpine 5 0,6 mg 12 hr interv	6	19±5	209±9	46±13	94±5
Reserpine 9 0,6 mg at 12 hr interv	5	25±5	211±12	28±3	84±4
Controls	7	63±12	245±13	267±29	203±11

N distinct changes occurred in the duodenal mucosa. With the exception of one rat which was treated for four days, lesions were encountered in the mucosa of all the other reserpinized rats.

3 *Effect f reserpine on the gastric mucosa fter dexamethasone treatment*

The pretreatment with dexamethasone consisted of injections 6×2 mg, at intervals of 12 hrs, followed by th reserpinisation, also at ntervals of 12 hrs. Th rats were given eserpine, 4×2 mg/kg and were decapitated 5 hrs after the last injection. The controls were orrespondingly injected with 0,2 ml of saline.

TABLE IV. Count ± SE of the epithelial mitoses and mast cells, as well as number of stomachs with mucosal ulcers of reserpinized rats pre-treated with dexamethasone

Treatment	Number of rats	Mitoses	Mast cells	Stomachs with ulcers
Reserpine 4 × 1.0 mg at 12 hr interv	15	52 ± 6	153 ± 25	12
Dexamethasone 6 × 2,0 mg and reserpine 4 × 1.0 mg	1	137 ± 15	1 ± 0.2	*0
Controls	19	62 ± 3	753 ± 58	0

* Digested blood in 5 stomachs content.

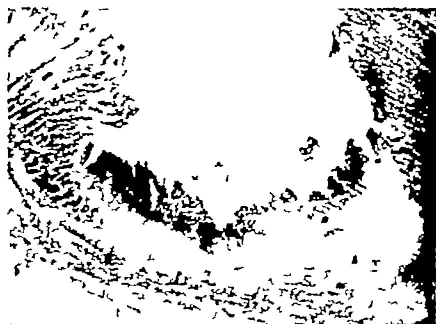


Fig. 4. Large reserpine ulcer in the gastric body mucosa. Magnification 40 ×

The mere reserpinisation distinctly degranulates (P<0.001) the mast cells of the gastric mucosa (Table IV). In mitotic count there was an insignificant decrease in this phase. After reserpinisation following pretreatment with dexamethasone the mast cells of the gastric mucosa were almost completely degranulated. On the other hand the mitotic count of the gastric epithelium was distinctly elevated (P<0,001).

In 12 rats which received reserpine exclusively ulcerations (Fig. 4) could be seen in the mucosa of the gastric antrum and body with the naked eye. In rats reserpinized after the dexamethasone treatment no ulcerations were found in the gastric mucosa. Old and digested blood was encountered in 5 ventricles and this probably was ascribable to the mucosal lesions induced by the preceding dexamethasone treatment.

Discussion

Reserpine in as low a concentration as 1×10^{-8} releases serotonin in the neoplastic mast cells in which the rapidity of the tryptophan transformation into 5-hydroxy tryptamine is dependent on the integrity of the granules (Carlini, Fischer and Giarman 1964) The concentration of serotonin in human blood decreases by half partial gastrectomy (Schmith *et al* 1959) The turn over rapidity of serotonin (Udenfriend and Weissbach 1958) and histamine (High, Shepherd and Woodcock 1963) in the gastric mucosa is highest.

Evidently as a primary phenomenon during the degranulation phase in the gastric mucosa of the rat a dilatation of the subepithelial capillaries occurs and these as well as the epithelium will be affected by lesion (Räsänen 1963 Guth and Hall 1966) The vagal stimulation induced by hypoglycemia with extreme rapidity degranulates the mucosal mast cells of the ventricle, causing large lesions in the gastric mucosa (Räsänen 1963)

After degranulation of the mucosal mast cells the local existence of vasoactive amines, equally the possibility of their local synthetizing might be insignificant. This is probably the cause preventing the ulcerogenic effect of reserpine after the dexamethasone treatment.

The superficial epithelium of gastric mucosa regenerates within about 24 hrs and in the same time a re-epithelisation of the lesions occurs in the glandular portion of the stomach of the rat. During the degranulation effect exerted by reserpine on the mast cells the mitotic count decreased in the gastric mucosa. However if degranulation of mast cells had been performed in advance by dexamethasone, there was a sharp increase in the number of mitoses in the gastric mucosa, without the following reserpinisation being able to decrease it. The desensitization of gastric mucosa is probably the result of the loss of local histamine and serotonin. The overstimulation of the stomach with compound 48/80 causes a rise in the mitotic activity of the gastric epithelium (Hunt and Hunt 1961)

The changes of the mitotic counts occurring in the study will lenghten the time of regeneration of the gastric mucosal epithelium from the normal one day up to 6 days. On the other hand during the mitotic overshooting time there is shortening of the regeneration to a third, or *8—10 hrs*. Probably the inhibition of mitoses renders continuation of the lesions, especially in an acid milieu where the decrease of the RNA polymerase and DNA-polymerase activity will become more marked owing to the heparin effect (Roth 1953) Thus is understandable the ulceration of the isolated antral mucosa during the histamine-reserpine stimulation after acidification Smith and Howes 1964 Howes *et al* 1965) The alkalisation of the gastric content will prevent the mucosal ulcerations due to th same mechanism in restraint rats (Sanyal, Banerjea and Das 1965) *After th dexamethasone treatment* the mucosa is preserved intact in spite of the reserpinisation, since there are no amines to be mobilized in the mucosa and the lesions produced during the preceding dexametha sone treatment are covered with new epithel during the mitotic overshooting

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Trapping of Sodium, Potassium, Sucrose, and Albumin in the Packed Cell Column of the Hematocrit

By

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Abstract

FUNDER, J and J O WIETH. *Trapping of sodium, potassium, sucrose and albumin in the packed cell column of the hematocrit.* Acta physiol. scand. 1967 *71* 105—112.

The observation of Maizels and Remington (1959) that high molecular markers are trapped to a significantly lower degree between centrifuged red cells (RBC) than small sized molecules, has prompted a study of the trapping of ^{131}I-labelled albumin, sucrose-^{14}C, ^{24}Na and ^{42}K. Albumin was found to be trapped to a significantly lower degree than sodium. The difference between trapping of ^{24}Na and of ^{131}I-labelled albumin at 0° C averaged 1.8 per cent, independent of the force and duration of centrifugation. Removal of membrane neuraminic acid had only a slight effect on trapping of albumin. The percentual trapping of ^{24}Na and of ^{42}K was found to equal trapping of sucrose-^{14}C, when the rate of Na and K uptake into RBC at 0° C was considered. These rates of sodium and potassium influx at normal extracellular concentrations were 0.24 and 0.027 meq/kg RBC/h. Based on the experimental findings the existence of a truly intracellular rapidly exchangeable fraction of RBC sodium is rejected.

A precise knowledge of the amounts of Na and K trapped between isolated cells is indispensable for the assessment of true cellular electrolyte concentrations and for the interpretation of isotope kinetics. The findings presented in this article have been the basis for the corrections employed in following works (Funder and Wieth 1967 a, b).

Clarkson and Maizels (1955) reported that human red cells (RBC) contain a Na fraction of about 3 meq per litre cells which exchanges immediately with the sodium of the external medium. Maizels and Remington (1959 a) found that the amount of easily exchangeable Na varied directly with external Na concentration, and found evidence that plasma markers of large molecular size (albumin and hemoglobin) were trapped to a significantly lower degree than those of smaller molecular size (lactose and inulin (Maizels and Remington 1959 b). This observation opened the possibility that the so-called easily exchangeable Na fraction of RBC is in fact located intercellularly.

In a previous work (Funder and Wieth 1966) it was emphasized that the great variability of reported normal values of RBC sodium can largely be ascribed to th

indiscriminate use of radioactive Na or labelled albumin for determining the amount of extracellular sodium analyzed with the cell specimens. Identical considerations have recently been advanced by Beilin et al. (1966) based on studies of trapping of ^{22}Na and of sucrose ^{14}C. Our studies confirm their observation that sucrose and sodium are trapped to a similar degree. It is further demonstrated that there is no difference between trapping of sodium and potassium. On the other hand trapping of albumin was constantly 1.5—2 per cent less than trapping of sodium, no matter whether the cells were packed very lightly by 5 min. centrifugation or whether they were subjected to ultracentrifugal forces (maximally 75 000 × g)

Methods

Experiments were carried out at 0° C to investigate whether the characteristics of trapped sodium and potassium are changed at a temperature which inhibits metabolism almost completely. Studies of the uptake of Na and K at 0° C confirmed that we largely avoided the difficulties arising from the uptake of sodium at higher temperatures (Beilin *et al.* 1966)

Samples of 10 ml of normal blood (pH 7 40 at 38° C) containing heparin (Boots) 0.125 mg per ml, were cooled to 0° C before addition of radioactive markers. The markers were added in 100 µl 0.15 M NaCl. After mixing the blood was immediately transferred to nylon hematocrit tubes (polyamid 11 length 93 mm, i. d. 3.5 mm) The tubes had a temperature of 0° C, being kept moulded in an ice cube. The capped tubes were submitted to centrifugation as described below. For the study of ^{22}Na and ^{42}K uptake at 0° C, tubes were kept in the ice for 3—5 hrs before isolating RBC.

Centrifugation was carried out at 0° C. Two centrifuges were employed. In one a relative centrifugal force of 6,000 × *g* (calculated at the bottom of the hematocrit tube) was obtained after 1 min acceleration. In the MSE Superspeed 25 centrifuge a maximum of 75,000 × *g* was obtained after 15—16 min acceleration.

Radioactive isotopes ^{131}I labelled human serum albumin was obtained from Philips Duphar (Amsterdam). The preparation (DRN 5305) contained 50 mg albumin per ml, specific activity 4 mC per g albumin. The potential source of error in determination of trapping of labelled albumin due to the presence of non-protein bound ^{131}I was stressed by Vasquez *et al.* (1952). In the batches received by us the range of free iodine was 0.9—2.2 per cent. Free iodine was determined by counting the ^{131}I found in the supernatant after precipitating ^{131}I albumin with 10 % (w/v) trichloracetic acid. By passing the albumin through a 15 cm column of a mixed-bed resin (Amberlite MB 3) the amount of non-protein bound ^{131}I could be lowered to 0 2 per cent. This result was not improved by repeating the ion-exchange, indicating either that a small fraction of the ^{131}I was not ionized or was liberated by the denaturation of albumin. The albumin preparation employed was dissolved in 0.15 M NaCl. Electrophoretic mobility of resin-treated albumin was identical with native albumin. The activities employed were 0 01—0 1 µC per ml of blood.

Uniformly labelled sucrose ^{14}C (Philips-Duphar Amsterdam) specific activity 70 mC per mmole was employed for determinations of trapping of sucrose. No impurities were found by autoradiography of thin layer cellulose chromatograms. The activities employed were 0.3—0.5 µC per ml of blood. Calculated sucrose concentrations in plasma were 0.01 mM

^{22}Na (Philips-Duphar Amsterdam) was obtained as chloride (specific activity 1 7 mC per meq Na). ^{42}K (AEK, Risø, Denmark) as chloride (produced by titrating irradiated K_2CO_3 with an equivalent amount of HCl). The specific activity of ^{42}K on delivery was 8 mC per meq. The activities employed per ml of blood were ^{22}Na 0.01—0.15 µC, ^{42}K 0 05—0.5 µC.

Trapping of albumin, sucrose ^{22}Na and ^{42}K was determined in separate samples from normal donors. ^{131}I, ^{22}Na, and ^{42}K were counted in a γ-well scintillation counter. ^{14}C was counted in a Packard Tri-Carb liquid scintillation spectrometer. 50 µl of the supernatant after precipitating approximately 300 mg of erythrocytes or 100 mg of plasma with 1 ml of 0 6 M perchloric acid were transferred to counting vials containing 10 ml of the scintillation medium (toluene 70 per cent (v/v) ethanol 30 per cent (v/v) 2 5-diphenyloxazole 3 5 g/litre, 1 4-bis-2 (4-methyl-5-phenyloxazolyl)benzene 0.07 mg/litre). Quench correction was found unnecessary. All samples were counted in duplicate.

Trapping of radioactivity was calculated in per cent of the activity of the external medium
trapped radioactivity = cpm/mg RBC × 100/ cpm/mg medium (per cent)

Uptake of ^{42}*K and* ^{24}*Na at 0° C* was determined simultaneously on samples to which both isotopes had been added. Influxes were calculated by dividing the increase in RBC activity with the specific activities of the medium. Since the specific activities of plasma were almost constant, the error introduced by not considering efflux of Na and K was negligible. The separation of ^{42}K and ^{24}Na by means of their differing half lives is described in following article (Funder and Wieth 1967).

Haematocrit (fractional RBC volume of one ml of blood) was measured on the evenly calibrated nylon tubes. The values were all within 0.35—0.50.

Treatment of red cells with neuraminidase was performed to investigate the importance of the N-acetyl-neuraminic acid of the red cell membrane for the trapping of ^{131}I-albumin. Neuraminidase isolated from culture filtrate of vibrio cholera was obtained from Behringwerke AG Marburg. The technique of neuraminidase treatment employed has been described by Eylar *et al.* (1962) Normal erythrocytes washed 4 times in 0.15 M NaCl were incubated one hour at 38° C with and without neuraminidase (the latter for control purposes) After incubation the cells were isolated by centrifugation and resuspended in plasma at haematocrit *of 0.30*. Thereafter determination of trapping of ^{131}I-albumin followed the procedure described above. It was controlled that N-acetyl-neuraminic acid was completely removed by the enzymatical treatment by comparing the amount liberated with the amount liberated from RBC after 30 min hydrolysis with 0.2 n H_2SO_4 at 80 C. Complete removal was found when cells had been washed prior to incubation, whereas the enzymatic liberation from unwashed cells was only 66 per cent of the total amount. Chemical determination of N-acetyl-neuraminic acid was made by the thiobarbituric acid method of Warren (1959)

Results

The percentual trapping of albumin, sucrose and sodium after ultracentrifugation of blood at 75 000 × *g* is shown in Table I Trapping of sucrose and sodium (2.1—2.3 per cent) was 1 6—1.8 per cent higher than the percentage of albumin (0.5) trapped between the cells. To evaluate whether the difference of 0.2 per cent between the values of trapped sucrose and trapped sodium is significant, determinations of trapped Na and sucrose were carried out simultaneously on paired blood samples from 8 donors. The results were Na 2.18 per cent, sucrose 2.10 per cent. The difference of 0 08 per cent equalled one standard deviation, so that Na and sucrose appear to be trapped to the same degree The correction for uptake of ^{24}Na at 0 C, which will be considered below is so small that it does not interfere with this conclusion.

TABLE I. Trapping of labelled albumin, sucrose and sodium. Blood samples were centrifuged at maximum of 75,000 *g* as described in method section. Duration of centrifugation 20 min

	Trapped radioactivity (per cent)	S E.	
I albumin	0.47	0 0	16
Sucrose-^{14}C	2.08	0 04	19
^{24}Na	2.27	0 01	42

TABLE II Effect of force and duration of centrifugation on the difference between trapping of ^{24}Na and ^{131}I albumin

Expt. Donor	Maximal relative centrifugal force during centrifugation	Duration of centrifugation (min)	Trapping (per cent) ^{24}Na (1)	^{131}I-albumin (2)	Trapping difference (1)—(2) per cent
2 XI 63	1 050 × g	5	24 42	22.60	1.82
J W	1 050 × g	10	11 02	9.18	1.84
	4,200 × g	20	4 53	2.37	2.16
17 XI.64 A.B.	75,000 × g	20	2.30	0.45	1.85
29 XI 65	6,000 × g	5	10.53	8.46	1.87
J F	35,000 × g	10	4 17	2.87	1.30
	50 000 × g	15	2 71	1.28	1 43
	75,000 × g	20	2.54	0.68	1.86
	75,000 × g	30	2.21	0.48	1 73
				Mean	1 76
					9
				S.E.	0.09

Table II shows that the difference between trapping of albumin and sodium of about 1 8 per cent was found independently of the degree of packing the cells, and independently of the time employed for the separation. It appears that the difference is found even when the packed cell column contains more than 20 per cent of plasma constituents. The phenomenon is thus not confined to packed cells, and it must be assumed that the shear of the cell surface is accessible to small molecules and ions, whereas the possibility of albumin to enter this space is restricted It was investigated how much trapping of ^{24}Na could be reduced. At a relative centrifugal force of 75 000 × g a minimal value of 1 6 per cent was reached after 2 centrifugations.

According to the findings of Eylar *et al* (1962) the zeta potential of the red cell membrane is largely caused by the free carboxyl groups of N-acetyl neuraminic acid. To examine whether the low trapping of albumin is related to the presence of neuraminic acid in the membrane we examined the effect of enzymatic treatment with neuraminidase on trapping of albumin. The N acetylneuraminic acid content of normal RBC determined on cells from 26 subjects was found to be very constant 0 63 mmole kg RBC^{-1} (S D 0 03) 1 88 mmole per kg RBC solids (S D 0 09) Trapping of ^{131}I albumin was determined as described in the method section, centrifuging neuraminidase treated and control cells at 6,000 × g for 30 min. The percentage of trapped albumin in the neuraminidase-treated samples was 1.37 (S E. 0 05 n = 3) to be compared with a control value of 1 18 (S E. 0.02, n = 5) The effect of neuraminidase is thus far too small to explain the much larger difference between the trapping of albumin and of small molecules.

TABLE III Trapping of labelled K between packed red cells. (The erythrocytes were suspended in the electrolyt media described in the text, ouabain concentration 3 10^{-4}M. The suspensions were centrifuged 20 min at 0° C t maximal relative centrifugal force of 75 000 × g)

Sample no.	Potassium in medium (meq × l⁻¹)	Trapped ⁴²K (per cent)	Trapped K (meq/kg RBC)
1	5.3	2.43	0.13
2	5.3	2.69	0.14
3	4.3	2 60	0.11
4	4.2	2.51	0.11
5	4.5	2.54	0 11
6	4.8	2.66	0.13
7	6.0	2.44	0.15
8	59	2.15	1.27
9	65	2.26	1 47
10	65	2.29	1 49
11	142	2.25	3 20
12	142	2.20	3.12
13	142	2 22	3.15
14	142	2.22	3.15
15	142	2.13	3.03
16	142	2.29	3.25
17	142	2.19	3.11
18	142	2.23	3 17

A series of experiments in which trapping of ⁴²K was determined is reported in Table III As t was found that trapping of ²⁴Na was not affected by substituting plasma with Ringers solution, trapping of ⁴²K was studied on RBC suspended in isotonic electrolyte media By these means the potassium concentration of th external medium could be varied between 4 and 142 mM. The electrolyte medium employed for samples no. 1 through 7 is the chloride medium" described in a following article (Funder and Wieth 1967 b) The media employed for samples no. 8 through 18 was of similar composition, with th exception that appropriate amounts of Na were substituted by K. To ascertain complet nhibition of ouabain sensitive uptake of potassium ouabain was added to the media in a concentration of 3 10^{-4}M When K concentration of the medium was above 50 mM as in expts. 8—18, the amount of trapped ⁴²K was always below 2.3 per cent. At K concentrations of 4 2—6 mM the percentage of trapped ⁴²K veraged 2.55 in 7 experiments. Evidence will be given below that th higher percentage found at low external K concentrations was due to uptak of labelled K int th cells. In the fa right column of Table III the alculated amount of labelled K found per kg cells is presented This amount of labelled K is directly proportional to external potassi m concentration. This is nalogous with the find ngs of Manzels and Remingt n (1959 b) that the ea ly ex hangeable sodium fraction varies directly with external Na concentration.

An ideal marker of extracellular substances must not be taken up by the cells. This claim was completely fulfilled by sucrose. The average value of sucrose found between cells packed after 21 hrs incubation at 0° C in sucrose-¹⁴C labelled plasma was 2.20 per cent (S.E. 0.03, n = 9) to be compared with a value of 2.16 (S.E. 0.05, n = 9) found by centrifugation of the blood immediately after addition of labelled sucrose. The average uptake of labelled sodium in 13 experiments at a plasma sodium concentration of 140 mM was 0.24 meq/kg RBC/hr (S.E. 0.01) equalling 0.72 meq/kg cell solids/hr. At a plasma potassium concentration of 4.1 mM the average uptake of labelled K in 10 expts. was 0.027 meq/kg RBC/hr (S.E. 0.004).

The amount of potassium taken up by the cells was thus 1/10 of the amount of sodium taken up by the cells during the same period. As previously mentioned the uptake of K will affect determination of percentual trapping considerably more than in the case of sodium. The uptake of Na and K during one hour at 0° C will thus increase determinations of trapped Na and K in a normal blood sample by respectively 0.17 and 0.68 per cent. Control experiments showed that virtually no exchange of ²⁴Na and K takes place between the trapped plasma and the supernatant plasma of a hematocrit. Erythrocytes stored at 0° C packed in the hematocrit tubes in contact with K and ²⁴Na-labelled plasma did not increase their contents of these isotopes during a 3 hr period. This means that the red cells during packing will only exchange Na and K with the decreasing fraction of plasma trapped between the cells. Since the time lapse from addition of isotopes until a high degree of packing has developed under the conditions employed in this study can be estimated to be maximally 1/3 hr, the maximal corrections for uptake of K and Na at normal plasma concentrations are 0.23 and 0.06 per cent — respectively.

If by far the main part of ²⁴Na is located intercellularly in a hematocrit centrifuged at 0° C, it should be possible to remove the radioactivity by washing the cells once in ice-cold saline. In two experiments we found that 86—93 per cent of the radioactivity was removed from the cells after washing the cells once.

Discussion

Our results confirm that albumin markers are trapped to a significantly lower degree than sodium. The presumption that almost all sodium trapped between packed cells at 0° C is extracellular has been strongly confirmed by the observation that the sucrose space and the sodium space in the packed cell column are identical. We have previously reported a difference of 2.1 per cent between trapping of sodium and albumin between packed cells (Funder and Wieth 1966). The slightly lower value of 1.8 per cent found in this work is ascribed to improvement of temperature control. The consequences of the trapping phenomena to the true values of intracellular electrolyte concentrations have been discussed in our previous work, and recently by Beilin *et al.* (1966). The latter investigators based their reasonings on a comparative study of trapping of ²⁴Na and sucrose-¹⁴C

Their results only exhib t slight differences from ours. Their finding of trapping of 0.2 per cent more sucrose than sodium was suspected to be due to the presence of decomposition products of labelled sucrose penetrating the cells. W th recently recrystallized sucrose they found 0.3 per cent less trapping of sucrose than of sodium. However their technique of adding the isotopes to blood at 37 C and applying an estimated correction for the uptake of sodium leaves the possibility that trapping of sodium has been slightly underestimated. We therefore believe that the rapidly exchangeable sodium fraction of 0.5 meq/kg RBC which was considered to be intracellular by Bellin *et al.*, is in fact extracellular sodium The results of Table III show that extracellular potassium is trapped to exactly the same degree as sodium and sucrose, when conditions impeding potassium uptake into the cells are provided.

The techniques employed have largely excluded trapping of labelled Na as a fallacy in previous tracer studies of Na exchange in red cells. Harris and Maizels (1951) washed erythrocytes in isotonic glucose before determining radioactivity and Na content of cell specimens. Glynn (1956) analogously washed red cells in Ringer s solution to free them of extracellular radioactivity Solomon (1953) determined the extent of trapping of extracellular material between the packed cells by means of Na. In contrast to this Gold and Solomon (1955) corrected values of labelled and total RBC sodium by means of determinations of trapped extracellular material carried out with I-albumin. Since the purpose of the latter study was to investigate Na fractions of varying exchangeability of RBC in vivo, it is obvious that the results were affected by including a fraction of extracellular Na in cell sodium This error has been estimated to correspond to about 30 per cent of the true sodium content of the cells.

It is thus evident that the different trapping of various extracellular markers must be regarded in the determination of intracellular Na, as well as in kinetic studies of ion exchanges through the RBC membrane The percentages of extracellular Na trapped at 0° C under well defined conditions of centrif gation vary but little (T ble I) Standard corrections may therefore be applied to correct results for the presence of extracellular Na and K between packed cells.

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Effect of Ouabain on Glucose Metabolism and on Fluxes of Sodium and Potassium of Human Blood Cells

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Abstract

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A technique for the study of red blood cell (RBC) metabolism, ensuring control of pH, is presented. Ouabain caused significant reduction of glucose utilization (—15 per cent) and of lactate production (—17 per cent) of human RBC incubated at 38° C and pH of 7 40. 80—85 per cent of potassium influx and 60—70 per cent of sodium efflux were abolished in the presence of ouabain. The amounts of N and K, normally transported by an ouabain sensitive transport mechanism were compared with the reduction of metabolism, which was found in the presence of ouabain. The reduction of adenosine triphosphate turnover (as estimated from the decrease of lactate production) was found to be equivalent to movement of 3 moles Na and of 2.5 moles K per mole ATP

In the original study of the inhibitory action of digitalisglycosides on the active transport of potassium in human red cells (RBC) Schatzmann (1953) found no reduction of the metabolism of cells, which were exposed to the action of cardiac glycosides. It is still a widely held view that the inhibition of ouabain-sensitive sodium and potassium transport in RBC, has no effect on the magnitude of glucose metabolism (Glynn 1964) However Murphy (1963) has reported that the rate of glucose metabolism is depressed by 15 per cent in the presence of ouabain. Since a 15 per cent reduction of the rate of glycolysis may be evoked by the mere reduction of pH of th medium by 0 10 units, convincing evidenc of an effect of ouabain on glucose metabolism can only be provided by carrying out experiments under careful pH control

The present paper reports the results of simultaneous studies of glucose metabolism and cation fluxes of RBC carried out under control of pH A significant reduction of metabolism in th presence of ouabain is demonstrated, and possibl relations between ctive transport of Na and K and the rate of glucose metabolism are considered.

Methods

50—75 ml of normal human blood, containing heparin (Boots) 0.125 mg (10 I.u.) per ml, was incubated at 38° C immediately after sampling. pH was adjusted to 7.40 by titration with CO_2.

In experiments designed to last more than 5 hrs glucose was added (100 mg per 100 ml of blood). Ouabain (strophanthin-g, Merck, Darmstadt) when employed, was added dissolved in autologous plasma to give a final concentration in the incubated blood of 3×10^{-4} M.

The incubation assembly was a modification of the gasometric pH-stat of Vestergaard Bogind (1961). pH of the blood was kept at 7.40 by continuous adjustment of carbon dioxide partial pressure. A flow of atmospheric air was continuously lead through the incubation vessel, the removal of CO_2 being compensated by carbon dioxide supplied through a magnetic valve (Danfoss EVJD 1) which was operated by an automatic titrator (Radiometer TTT 1a). pH control before and after experiments indicated that pH was kept within ± 0.005 pH unit of preset value. The gas mixture employed was 35 per cent CO_2 in atmospheric air. The incubation vessel was cylindrical (height 8.5 cm, diameter 6.5 cm). Incubation vessel, gas tubes, and humidifiers were all water-jacketed and thermostated at 38° C. The blood was continuously stirred by a 2.5 cm long, teflon coated magnetic bar. Glass electrode (Radiometer G 202 B) and calomel electrode (K 401) were employed for the continuous recording of pH of the incubated blood. To reduce leakage of KCl from the electrode (5—7 µeq per hr) the tip of the calomel electrode was fitted with a salt bridge filled with 0.15 M NaCl solution. Control analyses showed that potassium leakage was reduced below 0.1 µeq per hr. Electrodes were always allowed to equilibrate in plasma or Ringer's solution at least 18 hrs before the start of an experiment. Diffusion potentials, arising at the tip of the liquid junction, were stable after this time lapse and could be compensated after determining the pH of the incubated blood with conventional technique (Radiometer microelectrode E 5021).

The experiments on rate of glycolysis were of 3—7 hrs duration (average 5 hrs both in the absence and presence of ouabain). Samples of incubated blood were drawn anaerobically with hourly intervals. Plasma and RBC were isolated in nylon haematocrit tubes (Funder and Wieth 1966). The blood was immediately cooled to 0° C in the nylon tubes which were kept moulded in an ice cube. Centrifugation of blood was carried out at 0° C. Control investigations showed that no detectable glycolysis is found after one hours' storage of blood at 0° C. Red cells only take up small amounts of Na and K at 0° C. We found an uptake of 0.24 meq Na and of 0.027 meq K per kg RBC and hour at this temperature (Funder and Wieth 1967).

Analytical methods and calculations

Electrolyte concentrations and fluxes in RBC were calculated in meq per kg RBC solids, thereby eliminating errors derived from shifts of water between cells and plasma. The methods of determination of Na, K, water and solids in red cells have previously been reported in detail (Funder and Wieth 1966). Net fluxes of sodium were determined by flame photometric analysis of red cells. RBC sodium was corrected for the trapping of extracellular sodium between the packed cells (Funder and Wieth 1967). Net fluxes of potassium were calculated from the changes of plasma potassium concentration as well as determined directly by analyses of red cells. Potassium net flux could be calculated with a high degree of precision from plasma concentrations in contradistinction to the precision obtained by direct potassium analyses of the cells. This is simply due to the fact that net flux of potassium — even in the presence of ouabain — is maximally 2 per cent of the intracellular potassium content per hour.

Isotopes ^{24}Na and ^{42}K was added to the blood at the moment of initiating the experiments reported in Table VII. ^{24}Na (Philips Duphar, Amsterdam) was obtained as chloride with a specific activity of 1.7 mC/meq Na. ^{42}K (AEK, RISO, Denmark) was employed as chloride (produced by titrating irradiated K_2CO_3 by an equivalent amount of HCl). The specific activity of ^{42}K was 8 mC/meq K on receipt, and the isotope was employed for experiment within 48 hrs thereafter. In experiments we employed 0.05—0.15 µC ^{24}Na and 0.1—0.5 µC ^{42}K per ml of blood. The maximal increase in plasma potassium caused by addition of ^{42}K in experiments with transporting cells was 0.2 meq/litre.

Both ^{24}Na and ^{42}K were counted in a γ-well scintillation counter, the counting efficiencies being near 70 and 30 per cent respectively. Countings were carried out on samples of plasma, isolated lysed cells, and blood. The two isotopes were separated by means of their half lives. The samples were counted twice, the first time on the day of experiment, the second 8—10 days later. The activity of ^{42}K is reduced by a factor of 10^4 after 10.4 days, at which time more than 99 per cent of the ^{24}Na activity remains. ^{42}K activities determined by subtraction of the ^{24}Na activity were corrected for radioactive decay. Countings were extended sufficiently to give standard deviations of less than one per cent of the counts for each isotope.

Unidirectional fluxes were calculated from the net fluxes of Na and K, and from the transfer of [22]Na and [42]K to the cells. All calculations were based on the following relation between net flux and unidirectional fluxes

$$d(C_i)/dt = M_{in} - M_{out} = k_1(C_o) - k_2(C_i)$$

where C is the concentration of the ion considered (meq/kg solids), M_{in} and M_{out} denotes influx and efflux respectively (meq/kg solids h), k_1 and k_2 are the crude rate constants of influx and efflux, the subscripts i and o denoting inside phase (cells) and outside phase (plasma) respectively.

In the calculation of Na fluxes the advantage that the specific activity of extracellular Na remains constant was utilized, employing the equations derived by Clarkson and Maizels (1955) without modifications.

Unidirectional fluxes of potassium were calculated from the equation for a non steady state in two compartemental system (Sheppard 1962, p. 123), solving the slightly modified equation

$$a_i(t) = \frac{a_o(0)}{S} \cdot S_o(0) \left[1 - \exp\left(-\int_0^t k \frac{S_o \cdot S}{S_o \cdot S_i}\, dt\right)\right]$$

where the symbols have the following significations

$a_i(t)$ is the specific activity of potassium in cells at time t (cpm/meq)

$a_o(0)$ is the specific activity of potassium in the medium at the start of the experiment (= time (0)) (cpm/meq).

S is the total amount of K in the system considered (meq).

S_o is the total amount of K in medium (meq)

S_i is the total amount of K in cells (meq)

When calculating magnitude of fluxes S_i is normalized to be the amount of potassium found with 1 kg cell solids. k must accordingly be transformed to the rate constant k_1 by the relation $k_1 \cdot K_o = k \cdot S_o$; K_o being the potassium concentration of the medium (meq/kg).

The relation between K_o and S_o is given by

$$S_o = K_o\,(1-H)/(1-W) \quad (H),$$

W being the water fraction of the red cells, and H the weight haematocrit as defined in the following section.

Wet fiber of water was determined by drying cells to constant weight. Changes of that fraction of the cell suspension which was made up by external medium (plasma) could be determined from the relation

$$(1-H) = (\text{cpm/mg CS}) - (\text{cpm/mg RBC}) \,/\, (\text{cpm/mg M}) - (\text{cpm/mg RBC})$$

H being the fractional weight of red cells ("weight haematocrit"), CS = cell suspension, M = medium. Countings of [24]Na were employed for insertion into the above equation. The amount of external medium corresponding to one kg of cell solids was determined by the relation $(1-H)/(1-W)$ (H) as shown in the preceding paragraph. The relation between cell solids and plasma in normal blood with a haematocrit of 0.4 is approximately 4—5 kg plasma/kg cell solids. Initial intracellular Na content is thus only about three per cent of total sodium content of the suspension.

To be able to relate fluxes to area of cell membrane the following figures are useful. The mean value of the surface area of the red cell is 163 μ^2 (Ponder 1948, p. 24), the normal relation of cells to cell solids is 3 $\times$ 10[13] cells per kg solids (Funder and Wieth 1966 b). The surface area of the cells containing 1 kg solids is thus 4.89 $\times$ 10[7] cm². Fluxes expressed in mmole/kg cell solids h can accordingly be converted to mole/cm² sec. by multiplication with 0.57 $\times$ 10[-]

Glucose was determined in plasma by means of a glucose oxidase method after precipitation of proteins with $Zn(OH)_2$ *(Eriksson, Hjelm and Lindahl 1963). S.D. of the method (from 100 consecutive* double analyses) 0.047 mmole/l (0.84 mg per 100 ml). Mean and SE of recovery of glucose added to plasma was 99.7 per cent (SE 0.63, n = 8).

Glucose consumption of red cells (mmole/kg RBC solids h) was calculated from the disappearance in plasma utilizing the fact that glucose is evenly distributed between plasma and RBC water (Orskov 1946, Britton 1964). Accordingly glucose consumption (mol/litre blood per hour) — ΔG_B is

$$-\Delta G = (-\Delta G_p)\,(1-H) + (-\Delta G_p\, H\, W)$$

Where $-G_p$ is the disappearance of glucose in mmole/litre plasma per hour, H is the volume haematocrit fraction, and W is the fractional water content (v/v) of RBC.

The glucose consumption expressed as mmole/kg RBC solids per hour is

$$-\Delta G/H \cdot S$$

where S is the weight (kg) of the solids of one liter of RBC.

Plasma l(+) lactate was determined enzymatically employing l(+) lactic acid dehydrogenase (Lundholm, Mohme Lundholm and Vamos 1963)

S.D. of the method, calculated from the results of 100 consecutive double analyses, was 0.124 mmole/liter (1 12 mg per 100 ml) Mean and S.E. of recovery of lactate added to plasma was 99.6 per cent. (S.E. 0.49 n = 26)

Lactate production of red cells was calculated from the ppearance of lactate in plasma th consideration of the fact that the lactat ion is distributed according to the Donnan distribution of diffusible anions between cells and plasma. A distribution ratio of 0 662 t temperature of 38° C and pH of 7 40 was employed (Funder and Wieth 1966)) According to this lactate production (mmol /liter blood per hour) + ΔL_B is

$$+ \Delta L = (+ \Delta L_p)\ (1-H) + (\Delta L_p\ H \quad W\ 0.662)$$

where L_p is the increase of plasma lactate in mmole/liter plasma per hour and the other symbols have their previous meaning. The ensuing calculation of lactat production l mmole/kg RBC solids per hour is performed by multiplying ΔL_B with $1/H \quad S_1$

Direct determinations of lactate in blood were compared with the values calculated from determinations of plasma lactate. In the concentration range 3.8—6.1 mmole lactat /l blood the calculated lactate concentration was 100.6 per cent (S.E.0 7 n = 7) of the value found by direct determination.

The relation between glucose consumption and lactate production was evaluated by means of the lactat production index ($I_{lactate}$)

$$I_{lactate} = \frac{\text{mmole lactate produced}}{2 \quad \text{mmole glucose consumed}}$$

A lactate production index of 1 indicates complet conversion of glucose t lactate.

Hemoglobi concentration of blood was determined by colorimetric measurement of 1:251 dilution of blood in 0.04 per cent mmonia water (Haemotest, Copenhagen) The degree of hemolysis during experiments was calculated from the increases in plasma hemoglobin measured with sensitive benzidine reaction (Crosby and Furth 1956) In four experiments, each of 6—7 hours duration the average hemolysis (per cent hemoglobi liberated of total hemoglobin content) was 0.036 per cent per hour (T ble I) Hemolysis was not increased in the presence of ouabain.

TABLE I Hemolysis of blood during incubation with and without ouabai t 38° C (pH 7 40) Details about the incubation assembly are given in the *Method* section. The degree of hemolysis was calculated as per cent hemoglobin liberated per hour of total content of RBC

Expt	hours	Ouabain (M)	Hematocrit	Δ Plasma haemoglobin (g l⁻¹ h)	Hemolysis (per cent h⁻¹)
J.F 8.VI 64	6	0	0.43	0.085	0 035
E.B.B. 15.VI 64	6	0	0.47	0.104	0 034
J.F 11 VI 64	7	5 10⁻⁴	0.45	0.078	0.031
G.B.B. 8.IX.64	6	5 10⁻⁴	0.44	0.119	0.045

Results

Glucose Metabolism

Table II shows the glucose utilization and lactate production of normal and ouabain treated human blood cells The rate of glucose metabolism of the ouabain treated

TABLE II Effect of ouabain on glucose utilization and lactate production
Blood incubated at 38° C (pH 7 40) The results are given as means of two series of experiments comprising 12 and 7 normal donors. The standard error of the mean is stated in parenthesis. Net fluxes of sodium, potassium, and water are shown in Table III and IV

Ouabain (M)	Number of expts.	Glucose consumption (mmole kg solids^{-1} hr^{-1})	Lactate production (mmole kg solids^{-1} hr^{-1})	Lactate production index $\left(\frac{\text{mmole lactate}}{2 \text{ mmole glucose}}\right)$	g solids per l RBC	g hemoglobin per l RBC
0	12	4 77 (S.E.0.13)	9.63 (S.E.0.28)	1.006 (S.E.0.016)	381 (S.E.2.2)	348 (S.E.4.3)
3 10^{-4}	7	4.10 (S.E.0.16)	7.95 (S.E.0.40)	0.971 (S.E.0.041)	376 (S.E.5.2)	343 (S.E.3.3)
P		<0.01	<0.005	>0.40	>0.40	>0.40

cells was 15 per cent lower than that of unpoisoned cells. Lactate production was depressed by 17 per cent in the presence of ouabain. These differences are statistically significant ($p < 0\ 01$)

The contents of solids and hemoglobin of the cells are given in the right part of Table II Hemoglobin uniformly constitutes 91—92 per cent (w/w) of the cell solids, and the contents of solids in both groups were close to 380 g per liter RBC. Since metabolic data of RBC have usually been referred to cell volume or to hemoglobin content, it may be noted that the figures of Table II may be related to these parameters due to the constant relationships mentioned above.

The lactate production indices of the two series were close to 1 0 and the difference between them is not significant

Net fluxes of Na and K

Only minor changes were found in the concentrations of Na, K and water after incubation of normal cells (Table III) The cells exposed to ouabain gain sodium and lose potassium (Table IV) To determine the effect of the decreasing concentration gradients during incubation, both the results obtained after 1 hr lapse and the average net fluxes in the 0—3 hrs period of the 7 ouabain experiments are shown in Table IV The net fluxes of K, Na and water were 5—10 per cent lower when determined as an average of three hours incubation compared with the values found after the first hour Sodium gain is in both instances slightly greater than potassium loss, but the slight uptake of water by the cells does not differ significantly from zero The ratio of sodium gain to potassium loss was 1 1—1 2 in our experiments.

To determine the reproducibility of the determination of net fluxes of Na and K repeated determinations were performed on ouabain exposed cells from two donors. The results are shown in Table V

TABLE III Net fluxes of sodium, potassium and water in red blood cells (pH 7 40 38° C)
The results are given as means standard error of the mean and number of experiments are stated in parentheses. Initial Na and K content of RBC (meq kg solids⁻¹) Na 20.6 (S.E. 1 4) K 249.5 (S.E. 3.9)

Net flux		
Sodium meq kg solids⁻¹ h⁻¹	Potassium meq kg solids⁻¹ h⁻¹	Water g kg solids⁻¹ h⁻¹
—0.30	0.08	—6 0
(0 08, n=12)	(0.09 n=12)	(2.5, n=12)

TABLE IV Net fluxes of sodium, potassium and water in red blood cells in the presence of ouabain 3 10⁻⁴M. (pH 7 40 38° C)
The results are given as means standard error of the mean and number of experiments are stated in parentheses. Initial Na and K content of RBC (meq kg solids⁻¹) Na 19 4 (S.E. 1 7) K 258.5 (S.E. 6.3)

Period (hours)	Net flux Sodium meq kg solids⁻¹ h⁻¹	Potassium meq kg solids⁻¹ h⁻¹	Water g kg solids⁻¹ h⁻¹	Net flux Na / Net flux K
0—1	5 10	—4 18	3 43	1.22
	(S.E. 0 45 n=7)	(S.E. 0.46, n=7)	(S.E. 6.9 n=7)	(S.E. 0.13, n=7)
0—3	4.59	—4 05	2.81	1 13
average per h	(S E. 0 24 n=7)	(S.E. 0.25, n=7)	(S.E. 5.4 n=7)	(S E. 0.04, n=7)

TABLE V Reproducibility of the determination of net fluxes on red blood cells from two donors. Red blood cells incubated at 38° C (pH 7 40) in the presence of ouabain. In two of the J F experiments and in one of the J W experiments the red cells were suspended in Ringer solution of electrolyte composition as normal plasma. (cf chloride medium, Wieth and Funder 1967 b)

	Initial content meq kg solids sodium		potassium		Sodium net flux meq kg solids⁻¹ per 3 hrs		Potassium net flux meq kg solids⁻¹ per 3 hrs	
Donor	J F	J W	J F	J W	J.F	J W	J F	J W
Mean	19.5	1 4	2(3 5	257.8	12.5	12.02	—12.32	—10 64
S E.	0.6	9	3	4.3	0.40	0.43	0.29	0.25
( )	(5)	4	5	(4)	(5)	(4)	(5)	(4)

Relation of Transport of Na and K to Metabolism

Transport of Na and K against electrochemical gradients through the red cell membrane are considered to be closely related to the Na K sensitive adenosine triphosphatase activity of the membrane (Sen and Post 1964) Energy for the transport is delivered by hydrolysis of ATP produced by glycolysis in the cells. If the reduction of glucose metabolism in the presence of ouabain is caused by the abolishment of the energy requirement of the active cation transport, the relation between transport and metabolism may be evaluated from our data.

According to the results shown in Table II glucose can be considered to be almost quantitatively converted to lactate. In the Embden-Meyerhof cycle there is a net gain of one mole of ATP per mole of lactate produced. In table VI the net difference in ATP production in normal and in ouabain treated cells has been calculated from the difference in the rate of lactate production. Thus calculated the reduction of ATP turnover in the presence of ouabain was 1 7 mmole per kg RBC solids per hour

The magnitude of active Na and K transport was estimated from the net fluxes found in the presence of ouabain (Table IV) It is generally agreed upon (Sen and Post 1964 Whittam and Ager 1965) that initial net fluxes of Na and K in the presence of ouabain equals the amounts otherwise transported actively since sodium influx and potassium efflux are not affected by cardiac glycosides. Table VI shows that if the first hour s net flux are thus related to the decrease in ATP turn-over 3.0 moles Na and 2.5 moles K are transported at the expense of one mole of ATP

TABLE VI Estimation of the energy (ATP) utilization by ouabain sensitiv N and K transport. ATP turn-over was calcula ed from the reduction of lactate formation found in the presence of ouabain (T ble II) Net fluxes of N and K in the presence of ouabain (T ble IV) were considered to represent the magnitude of ouabain sensitiv transport. For further explanation see text

Difference in lactate production (T ble II) mmole kg solids^{-1} h^{-1}	Estimated difference in ATP turn-over mmole kg solids^{-1} h^{-1}	Net flux 0—1 h (T ble IV) mmole kg solids^{-1} h^{-1}		Relation of net fluxes ATP production	
		N	K	N /ATP	K/ATP
1.68	1.68	5.10	4 18	3.0	2.5

Unidirectional Fluxes f N and K

The measurements of unidirectional fluxes (Table VII) have at large confirmed preexisting information. Never the less the results f the six experiments in Table VII are hown to stress the fact that an appreciabl efflux of sodium and influx of potassium remains in the presence of ouabain. These fluxes were approximately

40 per cent (Na) and 15 per cent (K) of the values seen in the absence of glycosides.

This is of importance for the appreciation of the fact that the ratio between the net fluxes of Na and K in ouabain exposed cells differs from the ratio of the unidirectional fluxes of Na and K in unpoisoned cells. The ratios between sodium efflux and potassium influx in the transporting cells are in all 3 expts. between 1.45—1.50 indicating a ratio of sodium efflux to potassium influx of 3:2. The ratios of the net fluxes of Na and K in the presence of ouabain in the three experiments shown in Table VII were 1.15, 1.11 and 1.25. This agrees with the mean value of 1.13 of all seven experiments shown in Table IV.

TABLE VII. The effect of ouabain on unidirectional fluxes of Na and K in red cells.
All experiments were carried out at pH 7.40 (38° C). In the three lower experiments of this Table ouabain was added to the blood. M_{in}, M_{out} and M_{net} indicate influx, efflux and net flux.

Experiment	Ouabain (M)	Sodium: meq per l plasma	M_{in} meq	M_{out} kg solids^{-1}	M_{net} h^{-1}	Potassium: meq per l plasma 0 h	3 h	M_{in} meq	M_{out} kg solids^{-1}	M_{net} h^{-1}
J.F. 27 X.64	0	137	7.68	8.04	—0.36	4.95	4.50	5.43	4.81	0.63
J.W. 29.IX.64	0	141	6.65	6.65	0	4.60	4.30	4.57	4.17	0.40
G.B. 6 X.64	0	135	6.64	6.90	—0.26	3.50	3.10	4.69	4.48	0.21
J.W. 10 XI.64	3 × 10^{-4}	137	7.01	3.01	4.00	3.90	6.70	0.65	4.14	—3.49
K.M. 17 XI 64	3 × 10^{-4}	134	7.21	3.22	3.99	3.70	6.20	0.92	4.51	—3.59
A.J. 1 III 65	3 × 10^{-4}	130	7.84	3.10	4.74	4.90	7.10	0.93	4.69	—3.78

Discussion

Glucose Metabolism. Murphy (1963) reported a decrease of glucose utilization of RBC incubated at 37° C in plasma at a pH of 7.50 for 20 hrs in the presence of 10^{-4}M ouabain. The reduction was of the same magnitude as found by us, 0.3 mmole glucose per l RBC per hr. However pH was reported to fall up to 0.15 pH units in the experiment of Murphy. This fact might invalidate his results. The need for consta pH experiments on glycolysing blood cells is appreciated from the fact that he m author found a linear interdependence between the rate of glycolysis and pH the range 7.10—7.80, glucose utilization being doubled by the rise of pH of 0.7 Murphy 1960).

The present results demonstrate a significant reduction of glucose utilization and of lactate production of 15—17 per cent, when fresh blood cells, incubated in plasma at 38 C and at a pH of 7 40 are exposed to ouabain. The experimental conditions ensure that the differences found can not be due to differences in pH of the medium.

Lactate production index was found to be close to 1 in both groups (Table II) indicating an almost quantitative conversion of utilized glucose to lactate. This may seem surprising at first sight as RBC are known to have some aerob c metabolism of glucose in the phosphogluconic pathway By means of ¹⁴C glucose it has been determined that 11 per cent of the glucose molecules consumed enter this pathway (Murphy 1960) This means that 11 moles of CO are formed per 100 moles glucose. However since recycling in the phosphogluconic pathway is minimal, the pentoses formed after liberation of CO will be metabolized in the Embden Meyerhof cycle. Only about 2 per cent of the carbon atoms in metabolized glucose (11 of the 600 present in 100 molecules of glucose) will end up as CO_2, and the theoretically correct lactate production index is accordingly 0.98 Whereas the ratio of lactate to ATP formation in the Embden Meyerhof cycle is 1 1 the yield of ATP by the breakdown of ribose phosphate has been calculated to be 1 6 mole of ATP per mole of lactate formed (Sen and Post 1964) Since our calculations have been based on a production of one mole of ATP per mole of lactate, ATP formation may hav been slightly underestimated.

Unidirectional fluxes The valu of steady state exchange of Na in normal erythrocytes is 2.5—3 *meq Na per liter RBC per hour (Whittam 1964) At a solid con*tent of 381 g per liter RBC (Table II) the above figure corresponds with an exchange of 6.5—8 meq Na per kg RBC solids per hour in agreement with our results (Table VII) Reports of a steady state exchange of 1.5—2 meq K per liter RBC per hour (Whittam 1964) (4 0—5.3 meq K per kg RBC solids) are also consistent with the present esults The ratio of sodium efflux to potassium influx in transporting cells is thus about 3 2 However t must be stressed that this ratio cannot be accepted to be valid for the active transport of sodium and potassium, as long as the nature of the possible various components of sodium efflux and of potassium influx have not been defined.

Our finding of a potassium influx of 0 8—0 9 mmole per kg RBC solids per hour in the presence of ouabain 3 × 10⁻⁴M, a concentration which exceeds the concentration necessary for f ll inh bition f uabain-sensitive potassium transport by a factor of 100, is in agreement w th previous observations by Solomon, Gill and Gold (1957) and by Glynn (1957) The persisting efflux of Na n the presence of cardiac glycosides has been stud ed by Glynn (1957) who in 4 experiments found an efflux of 0 6—0.9 mmole per hour per liter RBC. At a sodium concentration of 8—10 mmole per liter ed cell this alue of the sodium efflux is consistent with a rate constant of N efflux (k_4) of about 0.1 hr Simila alues were found by Hoffman and Kregenow (1966) Th alues of k_4 found in the present experiments were 0 12 0 14 and 0 10

In accordance with these figures we found an efflux rate constant (k_e) of 0.14 (S.E. 0.01 n = 5) in a study of sodium efflux from human RBC suspended in Ringer's solution at 38 C and a pH of 7.40 (Funder and Wieth 1967 b).

By means of flux ratio analyses it has been concluded that the influx of potassium and efflux of sodium seen in the presence of ouabain cannot be interpreted as independent, passive fluxes (Whittam 1964). Exchange diffusion mechanisms of the type originally described by Ussing (1947) has been thought to be involved in both sodium and potassium fluxes (Glynn 1956). Recently it has been shown that the main part of ouabain insensitive sodium efflux is abolished in the presence of ethacrynic acid (Hoffman and Kregenow 1966). Experimental evidence was presented against the concept that part of sodium fluxes in red cells is mediated by exchange diffusion. Hoffman and Kregenow believed that the effect of ethacrynic acid was to abolish an active transport mechanism, which they named "pump II". Decisive proof of the existence of a second active transport mechanism has not yet been delivered, as it has not been possible to demonstrate a net movement of sodium against the electrochemical gradient in the presence of digitalis glycosides. In the case of potassium, exchange diffusion has not been ruled out, but in a following article it is shown that ouabain insensitive potassium influx only disagrees with calculated flux ratio in the presence of chloride and bromide ions (Funder and Wieth 1967 b).

The magnitudes of sodium influx and potassium efflux, the fluxes which are directed down the electrochemical gradient, were not changed appreciably by the presence of ouabain (Table VII). This is the background for accepting net-fluxes of Na and K in the presence of ouabain as indicative of the magnitude of active transport.

Net Fluxes of Na and K

The accuracy of the determination of the net fluxes depends on the chemical method employed. Details about the flame photometric method employed in this study have been presented elsewhere (Funder and Wieth 1966 a). The recovery of sodium and potassium added to lyzed cells were 100.5 per cent (coefficient of variation 1.9) and 100.0 per cent (coefficient of variation 0.8). The standard error of repeated determinations of net fluxes in cells from the same donors (Table V) were found to be 1.2 to 1.3 of the standard errors found by the determination of net fluxes in cells from 7 donors (Table IV).

Previous work on net fluxes of Na and K in the presence of ouabain has recently been discussed (Sen and Post 1964). There seems to be a tendency to anticipate that the ratio M_{out} Na/M_{in} K should be either 1.00 or 1.50 indicating the stoichiometrical relation between active transport of Na and K. It is apparent from our results (Table IV and VII) that although the unidirectional fluxes of Na and K bear a relation of 1.5 : 1 to another, the suppression of sodium efflux and of potassium influx in the presence of ouabain results in net fluxes of Na and K with a mutual stoichiometrical relationship approaching one.

Relation of Ouabain Sensitive Sodium and Potassium Transport to Metabolism

From studies of the release of inorganic phosphate and net fluxes of Na in RBC ghosts produced by reversible haemolysis the relation of Na transport to ATP hydrolysis has been reported to be 3 meq Na transported per mmole ATP split (Glynn 1962) Similar results were obtained by Sen and Post (1964) In their study the turnover of ATP in ghosts rich in Na and ATP was calculated from the disappearance of labile phosphate (ATP) and the production of lactate and pyruvate A considerable formation of lactate from adenosine monophosphate was observed and accounted for in the calculation of ATP turnover Active transport of Na and of K was estimated from differences of net fluxes in transporting and ouabain treated ghosts. The average relation of Na transport to ATP hydrolysis in 6 experiments was 3 7 meq Na transported per mmol ATP In the presence of glycolytic inhibitors (N-ethylmaleimide, oxamate and iodoacetate) where energy for ion transport was solely delivered by hydrolysis of ATP the Na/ATP ratio in six experiments was 3 1 The relation of K transport to ATP hydrolysis was 2.4 in both experimental series.

We have found only a single report dealing with the relation between metabolism and sodium transport in fresh intact erythrocytes (Murphy 1963) From the data given a Na/ATP ratio of 4 6 may be calculated. However the values of sodium net flux found by Murphy exceeded those shown in table IV by about 3 meq per kg cell solids and hour If this is considered the Na/ATP ratio will be reduced to 2.9

Whittam and Ager (1965) studied the relation between active Na and K transport and ATP hydrolysis at varied intracellular Na and K concentrations. In experiments with intracellular Na concentrations ranging from 19—101 and K concentrations from 79—12 meq/l red cells, Na/ATP ratio was 3.2 and K/ATP ratio was 2.4 Our results thus agree with those of Whittam and Ager suggesting that the energy requirementes of the transport mechanism was not affected by the extensive procedures which preceded their experiments. The present results further suggest that the ouabain sensitive sodium and potassium transport of normal red cells has the same energy requirements as the transport of reconstituted erythrocytes supplied with high concentrations of Na and ATP

A stochiometrical relationship of transport of 3—4 mole N per mole ATP has been observed in a series f differing tissues including frog skin, toad bladder lens tissue, and the salt gland of the herring gull (review see Bonting 1964) In these tissues ATP is generated by oxidative phosphorylation. The results obtained on red cells indicate that Na transport can be sustained by the anaerobic glycolytic cycle with expenditure of the sam amount of energy

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Acta physiol. scand. 1967 71 125—126
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Distribution of Injected L-3,4-Dihydroxyphenylalanine (L-dopa) in the Adult Rat Testis and Epididymis

By

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Previous studies have shown that adult and puberal rat testis is very weakly stained shortly after administration of some vital dyes, while the excurrent ducts are heavily stained (Kormano 1967) These observations suggest the existence of barrier mechanisms in the testis which prevent some substances from being carried into the seminiferous tubules and to a great extent even into the interstitial tissue.

After administration of L-dopa, brain capillaries accumulate dopamine, the formaldehyde-induced fluorescence of which can serve as a histological marker of the blood-brain barrier to dopamine (Bertler *et al.* 1964) A free passage of L-dopa in tissues outside the blood-brain barrier has been reported (Bertler *et al.* 1964) although extraneuronal binding may influence the localization (Hamberger *et al* 1967) The present study of the blood-testis barrier was carried out by using L-dopa as a marker

A method used by Bertler *et al.* (1964) was adopted. The rats received an i. or i.p. injection of L-dopa (Fluka, 100 mg/kg) in saline 10—40 min before decapitation. Some animals were subjected to i.p. injection of nialamide (Niamid, Pfizer) and/or NDS-1015 (Smith and Nephew) 1 hr before L-dopa injection. Untreated animals served as controls. Frozen dried pieces of testes and different parts of epididymides, as well as pieces of brain and muscle tissue as control tissues, were exposed to formaldehyde vapour at + 80 C for 1 hr and embedded in paraffin wax directly under vaccum or after immersion in xylene. Deparaffinized 5 μ sections were studied with fluorescence microscope for the cellular localization of fluorescence.

No monoamine fluorescence was seen in the control testes and caput epididymides, which are devoid of adrenergic nerve terminals (Norberg *et al* 1967) Corpus and cauda epididymidis contained fluorescent nerve fibers around the ducts. In L-dopa treated animals the result was not appreciably influenced by the mode of injection or the post-injection time The testes showed intense fluorescence in small blood vessels and a weaker fluorescence was seen in the interstitial tissue. The intensity of interstitial fluorescence varied in its different parts. Some fluorescence was confined to the tubular basement membranes, but definitely no specific fluorescence appeared inside the seminiferous tubules (Fig 1)

A very different distribution of fluorescence was seen in the head of the epididymis. The whole intertubular connective tissue of the epididymis showed a moderate fluorescence The duct epithelium, on the other hand, was heavily fluorescent in the caput epididymidis (Fig) but the intensity of specific epithelial monoamine fluorescence declined towards cauda epididymidis, being positive in all segments, however Pretreatment with nialamide or NSD-1015 had no influence on the localization of monoamine fluorescence in testis and epididymis Observations on brain and muscle tissue were similar to those of Bertler *et al* (1964)



Fig. 1 ... dopa administration (100 mg/kg). A moderate ... fluore... but not in the seminiferous tubules. × 260.
Fig. ... same animal as in Fig. 1. An intense fluorescence is seen ... 260.

... the seminiferous tubules seems to prevent the ... scent metabolites into the seminiferous tubules ... monstrated histochemically. An existence of a barrier ... formed by the seminiferous tubules is suggested ... (1967). The present findings provide further ... to the blood brain barrier, although ... a different nature. The epididymal duct seems ... Functional dissimilarities between the ... responsible for the observed differences in the ... of the epididymal duct epithelium. The ... testicular capillaries of the adult rat do ... into the interstitial tissue. In fact, even the ... capillary walls is not as effectively restricted as ... K... 196...

... grant from Yrjö Jahnsson Foundation.

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From the Department of Pharmacology University of G

The Presence of Dopamine in Human Gastric

By

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The amount of dopamine (DA) found in the urine of man Hellner 1951) and rat (see Leduc 1961) normally ex (NA) and adrenaline (A) Furthermore the amount of DA in urine (DOPAC and HVA) are much higher th (see Ruthven 1963) Thus the DA isolated from urine of the DA turnover in the body Several sources for this suggested. Certain types of chromaffin cells present in the of importance. Chromaffin cells in ruminants normally DA (Bertler *et al.* 1959)

Of particular interest are the findings that DA can be mast cells of the hamster ear (Adams-Ray *et al* 196) and cells in the gastro-intestinal tract (Enerbäck 1966, Håka Håkanson and Owman (1966) have shown that af tryptophan administration large amounts of DA or 5-hyd accumulate in the gastric mucosa.

Different types of laboratory food can affect the urinary levels of NA and A remain unchanged (Leduc 1961) decreased during starvation, but increased again when amounts of DA have been found in feces from rat (H son 1967) The above findings make it of interest present in gastric juice

Samples of gastric juice were collected from 6 patients ulcus ventriculi or ulcus duodeni Three of the patients given histamine 0.5 mg and one case with partial the two other cases received no drug Only patient gastric juice were utilized The fluorimetric according to Carlsson and Waldeck 1958) and NA according to Häggendal (1963

DA was found in the gastric juice in all the 4 insulin. The concentrations were 0.03 0.02, 0.00 juice respectively In the last patient (the insulin 0.015 µg per ml were also demonstrable In the

patients not receiving any injections no catecholamines were found. The identity of DA in the samples was verified by showing that the activating and fluorescence spectra were typical for DA, and that the elution pattern of the apparent as well as of authentic DA was the same on columns of strong cation exchange resin (Dowex 50 X8 W). The two patients with the largest volume of gastric juice collected during 1 hr had the highest DA concentration. The amounts of DA found in 1 hr were, in these cases, about 10 μg (histamine case) and about 50 μg (insulin case).

Further studies on normal men with and without drug treatment, e.g. insulin and histamine, appears to be necessary. The presence of free DA in the gastric juice makes it likely that the gastrointestinal tract may contribute materially to the total turnover of DA in the body. This gastric DA may suggest a local neurohumoral function of DA physiologically similar to that supposed by Holtz (1959) for the DA found in intestines.

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Muscle Glycogen during Prolonged Severe Exercise

By

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Abstract

HERMANSEN L., E. HULTMAN and B. SALTIN *Muscle glycogen during prolonged severe exercise* Acta physiol. scand. 1967 *71* 129—139

10 well trained and 10 untrained subjects worked to complete exhaustion on bicycle ergometer with work loads averaging 77 (76—87) per cent of their individual maximal aerobic power Determinations of glycogen used by working muscles (biopsy of lateral portion of the quadriceps femoris muscles) and of combusted carbohydrate ($\dot{V}_{O_2}$ and RQ) were performed at certain intervals from the start of work to exhaustion. At combustion rate of about 3 g carbohydrate per minute (RQ around 0.9 or higher) and at average values for glycogen in resting muscle of 1.6 (1.1—2.5) g/100 wet muscle, the effective work time was around 85 min for the untrained and 90 min for the trained subjects. At the end of the exhaustive exercise the glycogen content averaged 0.06 g in the untrained and 0.12 g/100 g wet muscle in the trained subjects. A close relationship between utilized glycogen and combusted carbohydrate was found, and it seems highly probable that high relative workloads primarily the glycogen stores in the exercising muscles will limit the capacity for prolonged strenuous work.

The relative role of different fuels utilized during various types and phases of muscular exercise has been widely discussed for about a century It is generally agreed that protein plays a non-essential role (Margaria and Foa 1939) In 1896 Chauveau showed that the ventilatory exchange ratio (V.E.R.=R.Q.) increased from 0.75 during rest to 0.95 during exercise, which he considered proved an increased utilization of carbohydrates during exercise but as the RQ was below 1.0 he concluded that fat was also used Zuntz *et al.* (1894) demonstrated that diet could markedly influence the RQ during rest and exercise and Krogh and Lindhard (1920) confirmed that observation. The question of whether or not the RQ during exercise is a valid indicator of the relative proportions of fat and carbohydrate used by the muscle during exercise was investigated by Christensen and Hansen 1939 a) On the basis of measurements of the CO and lactate content of the blood they concluded that the RQ measurement even at high but not maximal work levels after 10—15 min of exercise gave a true representation of the fuels utilized (true RQ Christensen and Hansen 1939 d) also demonstrated that the RQ was dependent on the work level, and well-trained subjects had a lower RQ at the same external work load compared

with untrained subjects. During prolonged exercise as well as after a high fat diet, the RQ was lower indicating increased utilization of fat (Christensen and Hansen 1939 b c) More recent studies have emphasized the role of fat as an important fuel for muscular exercise (for references see Rodahl and Issekutz 1964) During exercise the arterial concentration of FFA shows a transient decrease, followed by an increase (Friedberg *et al* 1960 and Carlson and Pernow 1961) which also represents an increased utilization of FFA. For example Havel *et al* (1963) have estimated that 41—49 of the energy output is derived from the direct oxidation of FFA during long sustained work compared to 25—26 / during rest. Furthermore Paul, Issekutz and Miller (1966) have shown, during prolonged exercise in dogs, that 90—95 % of the extramuscular sources for energy are derived from fat. In view of these recent observations the concept based on RQ-determination during exercise, that carbohydrates are the predominante fuel for heavy exercise has been questioned.

A muscle biopsy technique (Bergström 1962) permits a more precise determination of the glycogen utilization during exercise (Hultman 1967a Ahlborg *et al.* 1967) The aim of the present study was to compare the actual amount of glycogen utilized by an exercising muscle with the calculated carbohydrate usage based on the RQ and O uptake in an attempt to reinvestigate the concept that carbohydrate is the major fuel for strenous muscular exercise. As training might conceivably influence the energy utilization, the study was performed on both untrained and well-trained subjects.

Subjects

The subjects were 20 healthy males, age 20—30. Ten of the subjects had not performed regular training during the last 5 years, and their maximal oxygen uptake eraged 3.4 l/min (42—56 ml kg min) The remaining 10 subjects trained intensively t least couple of times per eek all ea round nd all competed endurance events. Their maximal oxygen uptak eraged 4 6 l mi (60—72 ml/kg min) The erage height of all he subjects was 179 cm and the weight of he ntrained subjects was 73 nd 67 kg for the trained subjects. I additional experiment, 8 physic l education students were studied. They were ll somewhat trained and their body eight a eraged 1 kg heigh 177 cm, nd maximal oxygen uptake 4 1 l/min.

Methods and procedure

Expired air for he det rmination of oxygen uptake was collected Douglas bags and the olume measured in spir meter (Saltin nd Åstrand 1967) The gas was analyzed ith modified Haldane technique Heart as determined by coun ng t least 30 R R intervals from ECG recordings. Lactate nd pyru er determined enzymatically on fi gertip blood (Scholz *et al* 1959) and glucose by the ortho toluidine method (Hultman 1959) The muscle glycogen content as determined ccording the me hod described by Hultma (1967) The subject's maximal oxygen uptake (Åstrand and Saltin 1 1 as well as check of their bili y for prolonged hea y exercise were performed coupl of da bef re he actual experiment On the experimen al day the subjects came to the laboratory in he morning af er ha ng had cup of offee or tea and single sandwich. No special instructions were g en for heir diet and physical acti ties he days preceding the experiment, but no one had ained h day before the study The subjects rested for t least 15 min in the laboratory before the basal determ na ions were made These included muscle biopsy for glycogen content, oxygen ptake heart ra blood lact pyru nd glucose The subjects

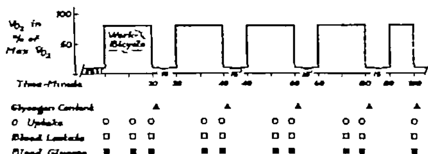


Fig. 1 The general procedure for the intermittent exercise experiments. For more detailed description see the text.

then started to exercise on bicycle ergometer (Krogh or Monark) with constant pedal frequency of 50 rpm at work load which averaged 77 (71—87) per cent of each individual' maximal oxygen uptake. Each 20 min period of exercise (I—V Table I) was followed by 15 min rest (see Fig. 1) The experimental design required that the subjects exercised to exhaustion. During the experiment the subjects had to drink 0 15% saline solution and they kept their body weight almost constant. Around the 11th and 19th minute of the exercise periods oxygen uptake and heart rat was determined and blood sampl was drawn from fingertip prewarmed in warm water for lactate, pyruvat and glucose determinations. Within the first couple of minutes of each rest period, the muscle biopsy was performed the biopsy needle and the technique used has been described by Bergström (1962) After cutaneous local anesthesia an incision was made in the skin and the needle as introduced into the deeper part of the lateral portion of the quadriceps. A new incision was made for each biopsy Usually three biopsies were made in each thigh, starting distally

I 4 subjects, (3 trained and 1 untrained) the prolonged exercise was repeated few weeks later similar manner as described above The only exception was that the second time no rest was permitted and the exercise was continuous until exhaustion. The incisions in the skin for the biopsies were all made before the exercise started The biopsies were then made at 20 min intervals with the subjects sitting on the bicycle the interruption in the continuous pedaling was limited t only 15—30 sec.

I he additional set of experiments the 8 physical education students performed three one-hour exercise periods weekly intervals work levels of bout 25, 50 and 80 per cen of their maximal oxygen uptake

Results

Tabl I summarizes the results after statistical treatment. The oxygen uptake was almost constant for the different work periods I—V throughout the experiment F g 2) The erage oxygen uptake for the untrained subjects was 2.8 l/min or 79 73—87 per cent of their maximal oxygen uptake and the corresponding figures for the tra ned group were 3 4 l min or 76 (71—80) per cent. After 11 min of exercise th heart rate was 164 for the trained and 172 for the untrained s bjects. The rate increased throughout the work period though never eaching maximal level Fg 2 I the untrained group th RQ during the first work period was 0 9(and dr pped to 0 93 at th end of the exercise Fig The RQ wa 0 91 after 11 min of exercise in the rained group and even after 80 mi of exercise was still 0 90 bu decreased to 0 88 during the last minutes of work Th carboh drat combusted during the exercise Fig 2 bottom was calculated from th erage oxygen uptake nd RQ each work period. The erage utilization of rboh drates wa for both groups

TABLE I. Average values, standard error of estimate, standard deviation and range for different
the untrained (U) groups. At rest and during the exercise periods I—IV the number
trained and 9 trained subjects

		Rest	I	II
		0.27±0.03	2.78±0.11	2.85±0.10
	U	0.08	0.36	0.32
Oxygen uptake		0.17—0.33	2.3—3.3	2.4—3.3
l/min		0.28±0.02	3.39±0.10	3.32±0.10
	T	0.06	0.31	0.32
		0.19—0.33	3.0—3.8	3.0—3.7
		0.83±0.01	0.96±0.01	0.94±0.01
	U	0.04	0.03	0.03
RQ		0.75—0.91	0.91—1.00	0.90—0.99
		0.82±0.02	0.91±0.01	0.90±0.01
	T	0.06	0.03	0.03
		0.74—0.92	0.86—0.97	0.84—0.99
		0.14	2.92±0.18	2.90±0.19
	U		0.57	0.59
Combusted		0.09—0.18	2.1—3.6	2.0—3.8
carbohydrates g/min		0.13	2.89±0.20	2.63±0.20
	T		0.63	0.62
		0.08—0.18	2.4—3.6	1.4—3.5
		1.58±0.10	0.66±0.11	0.44±0.11
	U	0.31	0.36	0.33
Glycogen content		1.25—2.21	0.25—1.48	0.04—1.09
g/100 g wet muscle		1.69±0.12	0.94±0.15	0.62±0.14
	T	0.39	0.40	0.44
		1.10—2.49	0.43—1.80	0.12—1.34
		93±2.6	83±3.4	86±3.4
	U	8.3	10.7	10.8
Blood glucose		81—110	62—97	69—107
mg/100 ml		87±2.5	82±3.3	85±4.2
	T	7.9	10.5	13.3
		78—101	63—100	69—106
		1.1±0.31	6.6±0.89	5.6±0.89
	U	0.5	2.1	2.8
Blood lactate		0.6—1.5	2.5—9.8	1.5—11.5
mM		0.9±0.28	5.1±0.79	3.7±0.60
	T	0.5	2.4	1.9
		0.5—1.3	1.7—9.2	1.5—7.2
		0.09±0.01	0.25±0.01	0.23±0.02
	U	0.02	0.04	0.03
Blood pyruvate		0.05—0.13	0.18—0.32	0.15—0.29
mM		0.08±0.01	0.21±0.02	0.19±0.02
	T	0.02	0.05	0.06
		0.05—0.12	0.15—0.30	0.10—0.26

parameters studied t rest nd during each work period to exhaustion for the trained (T) and of subjects are 10 in the trained and untrained groups. In exercise period V there are 7 un-

III	IV	V	Paired t-test
2.80 ± 0.11	2.78 ± 0.11	2.79 ± 0.08	
0.35	0.37	0.25	0
2.4—3.3	2.3—3.4	2.3—3.1	
3.36 ± 0 10	3.41 ± 0.11	3.37 ± 0.12	
0.32	0.33	0.37	0
3.1—3.6	3.0—3.8	2.4—4.0	
0 94 ± 0.01	0.93 ± 0.01	0.92 ± 0.01	
0.03	0.03	0.04	0
0.88—0 99	0.87—0.99	0.83—0.97	
0 89 ± 0.01	0.90 ± 0.01	0.88 ± 0.01	
0 04	0.04	0.04	0
0.83—0 99	0.83—0.99	0.83—0.99	
2.76 ± 0.20	2 78 ± 0.21	2.72 ± 0.24	
0.62	0 64	0.76	0
1.8—3.5	1.8—3.6	1.6—3.5	
2.62 ± 0 20	2.81 ± 0.21	2.58 ± 0.25	
0 63	0.65	0.79	0
1.8—3.3	1 7—4 0	1 4—3.2	
0.16 ± 0.05	0.07 ± 0.04	0.06 ± 0.04	R > I II, III, IV V
0.23	0 12	0.10	I > II III IV V
0—0.67	0—0 28	0 0—0 6	II > III IV V ; III V
0 24 ± 0 09	0 17 ± 0 07	0.12 ± 0 06	R > I II III, IV V
0.27	0.21	0.18	I > II III IV V II >
0 03—0.86	0 01—0 60	0.01—0.46	III IV V III, > IV V
85 ± 3 6	82 ± 2.2	82 ± 2.2	
11 3	6 8	6.2	0
71—101	72—96	72—91	
84 ± 4.2	81 ± 4.8	72 ± 5.7	
13.4	15 0	17 1	0
65—108	63—102	64—104	
4 6 0.79	3.5 ± 0.69	2.9 ± 0 71	I > III IV V
2 4	1	2 2	II > III, IV V
1 1—8 9	0 9—8.6	1 3—5.6	III > IV V
3 2 0 48	2 8 ± 0 45	8 ± 0 66	I II III IV V
1 5	1 4	2.0	II > IV V
1.3—5 8	1 1—5 5	0.8—6 2	
0 21 0 02	0 20 0 07	0 19 ± 0 01	I III IV V
0 03	0 06	0 04	II III IV V
0 14—0 28	0.09—0.29	0.14—0 26	
0 19 0 0_	0.18 ± 0 02	0.19 ± 0 02	I II III IV V
0.05	0 06	0 07	
0 13—0 23	0 12—0.26	0.09—0 24	

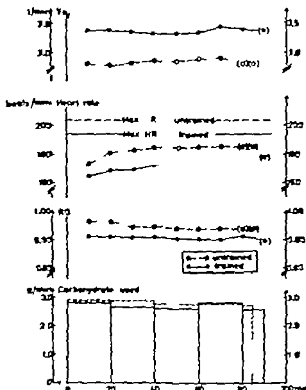


Fig. 2 A erage values for the 10 trained (filled symbols) and the 10 untrained (unfilled symbols) subjects for oxygen uptake ($\dot{V}_{O_2}$), heart rate, entilatory exchange ratio (RQ) and used carbohydrates during exercise t exhaustion. The symbols within parenthesis denotes 7 subjects (untrained) and 9 subjects (trained).

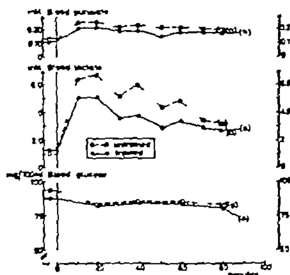


Fig. 3 A erage alues for blood pyruvat lactate and glucose at rest before exercise started and during the exercise to exhaustion in the trained and untrained group.

2.8 g min, and aried l ghth during the prolonged exercise. Paired t-test of intra individual differences n RQ and combusted carbohydrates showed no significant difference between th w rk periods Tabl I) The blood pyruvate concentration during work was slighth h gher in the untrained compared to the trained and the concentration remained in both groups fairly stable throughout the whole work

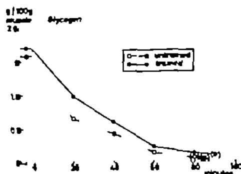


Fig. 4.

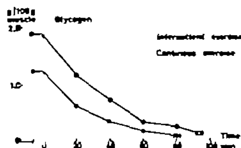


Fig. 5.

Fig 4 Average values for glycogen content in the lateral portion of the quadriceps muscle before and during exercise until exhaustion in the trained and the untrained group.

Fig 5. A comparison of the muscle glycogen content during prolonged exercise to exhaustion with 20 min work periods and 20 min rest (unfilled symbols) and continuous exercise (filled symbols) in 4 subjects. The symbols within parentheses denot only two subjects.

period (Fig 3) The blood lactate reached its highest level during the first work period and then declined (Fig 3) The untrained subjects average peak value was 6.8 compared to 5 1 mM in the trained. In both groups, at the end of exercise before exhaustion, the blood lactate concentration approached the resting level. Before work the mean blood glucose concentration (lower part Fig 3) was 93 in the untrained and 87 mg/100 ml blood in the trained group. At the end of the first 20 min of exercise t dropped to 83 and 82 mg/100 ml respectively and remained at almost constant level until exhaustion.

At rest (work time 0) the average values for the muscle glycogen content were 1.58 and 1 69 g/100 g wet muscle for the untrained and the trained respectively (Fig 4) The glycogen reduction was greatest during the first work period. Thereafter a slower removal rate was found, which was somewhat more pronounced in the untrained. Glycogen content averaged 0.06 in the untrained and 0 12 g/100 g muscle in the trained. In Fig 5 is shown the reduction of the muscle glycogen in continuous and intermittent work on the same work load in 4 subjects. The general appearance of the curve as well as the other parameters studied (HR, blood pyruvate lactat and glucose) were essentially the same during the intermittent and th con tinuous exercise even though the initial concentration of glycogen was lower in the *second set of experiments but the total work time was lso shorter*

An increase in work level from 28 to 54 per cent of the subjects maximal oxygen uptake increased th utilization of glycogen in the working muscle from 0 31 to 0.83 g 100 muscle per hour (Fig 6) When the average relativ work load was increased to 78 per cent of the maximal oxygen uptak the glycogen utilization rose to 1 56 g 100 g muscle for the one hour work period. Fig 7 demonstrates the rela tionship between the amount of carbohydrate combusted (estimated from $\dot{V}_{O_2}$ and RQ) during the exercise period and the amount of gl cogen utilized in the exercising muscle during the same period of time

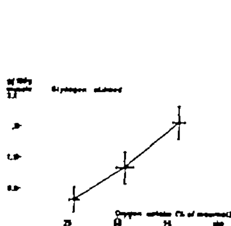


Fig. 6.

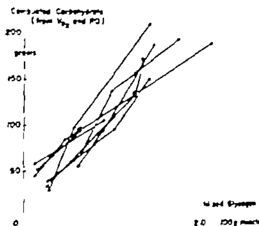


Fig. 7

Fig. 6. Decrease of muscle glycogen after 1 hr of exercise at three different work levels, mean ± SD, n = 8.

Fig. 7 Individual values for the amount of utilized glycogen during the one hour exercise period at different work levels in relation to the calculated amount of combusted carbohydrates.

Discussion

The subjects in this study performed strenuous exercise and worked to complete exhaustion. Among the parameters studied, neither oxygen uptake nor blood concentration of pyruvate, lactate or glucose ever reached values that revealed or parallelled the subjects' fatigue or explained the exhaustion. Probably dehydration can also be excluded as an explanation of the fatigue as the subjects' body weight reduction during the experiment was kept below 1 kg (Saltin 1964 part 2). The subjects were obviously tired at the end of each work period because of the high relative work level. However, they were unable to continue pedaling only when the glycogen content reached extremely low values. The rate of reduction of glycogen in the exercising muscle was most marked at the first exercise period. Thereafter being lower but relatively constant in two work periods and during the last work period very small. In contrast to this, the calculated amount of carbohydrate combusted was unchanged throughout the whole work and averaged 2.8 g/min.

It is worth emphasizing that the pattern of the glycogen decrease with time was the same during continuous exercise as when intermittent work was performed (cf. Fig. 5). The steeper fall in the muscle glycogen during the first work period (Fig. 4) may be explained by a greater production of lactate giving a greater accumulation of lactate in blood in the early phases of exercise. This is further shown in this series where higher blood lactate values and faster reduction in glycogen was observed in the untrained subjects compared to the trained.

A straight line through the values for the glycogen content at 20, 40 and 60 min of exercise probably gives the best indication of the glycogen removal due to aerobic processes. As the exercise proceeded to the period when the glycogen stores in the working muscles were almost emptied, extra muscular sources must supply some additional carbohydrate. If the calculated value of 2.8 g carbohydrate min is

correct. The only possible sources for this carbohydrate are the liver glycogen and the small amount of glucose in the extra cellular fluid. Rowell *et al.* (1965) have shown that the net glucose production from the splanchnic area during 60 min of exercise averaged 0.3 g/min. There is some evidence that during prolonged severe exercise the splanchnic production of glucose is low at the beginning of exercise but increases with time (Hultman, 1967b). If so, the amount of glucose available from the liver may account for the finding that the exercising muscle at a the liver may account for the finding that the exercising muscle at a very high relative work load combusts mainly carbohydrate also at the end of exercise, even though the muscle glycogen content is close to zero. It should be remembered that glycogen is determined only in the lateral portion of quadriceps, and RQ is determined for the whole body. This may mean that other muscle groups or other parts of the same muscle richer in glycogen gradually becomes more active. This seems, however, unlikely as the oxygen uptake during work and therefore the mechanical efficiency is fairly constant throughout the exercise period.

When exhausted, the subjects were able to resume work at a lower level of activity. The inability to perform heavy exercise was not due to a reduction in muscular strength, it could be shown in an earlier study that the maximal isometric strength was unchanged after prolonged exhausting exercise (Saltin 1964).

In the present experiments special care was taken to eliminate errors in order to get a true RQ. All subjects were very well aquainted with the experimental procedure and they had been subjects several times before. During exercise the collection of expired air started after 11 min of exercise. Frequently two determinations of the oxygen uptake were made consecutively and the RQs were always identical within the error of the gas analyses (Haldane). In spite of differences in blood lactate, variations in the measured RQ in each individual was very small (see Table I). Our impression is, therefore, that the calculated values for RQ accurately estimate the true RQ. This opinion is supported by the results presented in Fig. 7 showing a close relationship between utilized muscle glycogen and combusted carbohydrate calculated from the RQ. Furthermore in a similar study where the muscle glycogen content was varied over a wide range a very good relationship was found between the calculated value for combusted carbohydrate and the used glycogen, determined by direct measurement in muscle biopsy specimens (Bergström *et al.* (1967).

In Christensen and Hansen's experiment (1939b) the relative work load was around 60—70 per cent, and the RQ dropped from 0.88 to 0.80 during 180 min work. The same tendency with a decreasing carbohydrate combustion as work proceeded was found in Saltin and Stenberg's study (1964) in which the RQ dropped from 0.91 to 0.86 during 3 hrs exercise at relative work level of 72 per cent.

In contrast to these results Hedman (1937) found that during cross-country skiing for 150 min at constant speed with a relative work load of 80 per cent RQ was essentially constant from start to finish. The carbohydrate combustion was in this study close to 3 g/min, and the total consumption of carbohydrate was 445 g for the whole run. The results of the present study with relative work loads of 6 and 79

per cent are in agreement with these findings. At a relative work load of 76 per cent or higher the muscles obviously are not able to substitute carbohydrate with fat even when the available glycogen stores are emptied. At lower relative work loads exercise can be performed with a markedly lower utilization of glycogen in the active muscle (*cf* Fig 6) The average RQ:s were 0.87 0.90 and 0.93 at oxygen uptakes of 1.2 2.2 and 3.2 l/min respectively representing 29 53 and 78 per cent of the subjects maximal oxygen uptake. When the relative work load increased from 29 to 78 per cent the fat combustion changed only from 2.5 to 3.6 kcal/min compared to a change from 3.4 to 12.3 kcal/min for carbohydrate.

It is highly probable that at high relative work loads with RQ:s around 0.9 the glycogen stores in the exercising muscles is a decisive for maximal work time. Thus the capacity for prolonged strenuous work may be limited by the initial muscle glycogen content. Whether or not lack of glycogen in the muscle at the end of the exercise is the sole physiological determinant for the physical exhaustion can, however not be settled at present.

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Acta physiol. scand. 1967 71 140—150

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Diet, Muscle Glycogen and Physical Performance

By

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Recei ed 23 February 1967

Abstract

BERGSTROM, J L. HERMANSEN E. HULTMAN and B. SALTIN *Diet muscle glycogen and physical performance* Acta physiol scand 1967 *71* 140—150

The muscle glycogen content of the quadriceps femoris muscle was determined in 9 healthy subjects with the aid of the needle biopsy technique. Th glycogen content could be varied in the individual subjects by instituting different diets after exhaustion of the glycogen store by hard exercise. Thus, the glycogen content after fat + protein (*P*) and carbohydrate-rich (C) diet varied maximally from 0.6 g/100 g muscle to 4 7 g I all subjects, the glycogen content after the C diet was higher than the normal range for muscle glycogen, determined after the mixed (M) diet. After each diet period, the subjects worked on bicycle ergometer at work load corresponding to 75 per cent of their maximal O_2 uptake to complet exhaustion. The verage ork time was 59 126 and 189 min after diets P M and C, and good correlation was noted between ork time and the initial muscle glycogen content. The total carbohydra e utilization during the work periods (54– 790 g) was well correlated the decrease in glycogen content. I is therefore concluded that the glycogen conten of he working muscle is a determinant for the capacity t perform long-term heavy exercise Moreover has been shown tha the glycogen content and, consequently the long-term work capacity can be appreciably varied by instituting different diets after glycogen depletion

It was shown in previous studies that the muscle glycogen content decreased during work (Bergstrom and Hultman 1966 a) and that during exhaustive exercise the glycogen stores were almost completely emptied (Bergström and Hultman 1967). In a study by Hermansen, Hultman and Saltin (1967) it was found that the rate of combustion of carbohydrates was extremely high and constant throughout the whole work period In this study a well as in that of Ahlborg *et al.* (1967a) there were some indications that the initial muscle glycogen concentration was related to the ability to perform prolonged, heavy exercise (measured as the work time) provided that the subjects worked with the same relative load.

The muscle glycogen concentration in man can be considerably increased by first emptying the glycogen stores through hard work, and then giving a carbohydrate-rich diet. The enhancement of glycogen synthesis is localized to the muscles that have worked, and does not affect other muscle groups (Bergström and Hultman

TABLE I. Pertinent data in the nine subjects

Subject	Age yrs	Ht cm	B.W kg	Max. oxygen uptake l/min
T.P	22	179	76	4.93
B.T	22	176	69	4 03
Å.L.	26	173	62	3.37
R.S	20	174	75	3.94
S.P	25	179	67	3.77
S-O J	23	182	72	4.96
G.F	24	184	71	4.62
R.E.	23	173	62	3.57
K.-G.G.	24	177	73	4.46

1966 b) On the other hand, a fat + protein diet after exercise induces only a slow incomplete resynthesis of glycogen, and if carbohydrate is given without previous exercise, only a moderate increase in muscle glycogen takes place (Hultman and Bergström 1967) Thus, by varying the type of diet after exhaustive exercise, it is possible to obtain different muscle glycogen levels in the same individual.

As early as 1939 Christensen and Hansen showed that the capacity for prolonged exercise can be markedly varied by varying the subject's diet After 3—7 days of predominantly carbohydrate intake the work time was 210 min on a fixed load, compared to only 80 min after an equal time on a fat diet.

The aim of the present study was to determine the extent to which the muscle glycogen content could be altered in individual subjects by varying the dietary regime after depletion of the glycogen store, and subsequently to ascertain the relation between the initial glycogen content and the capacity for prolonged hard exercise

Material and methods

Nine physical education students participated in this study some pertinent data regarding them are given in Table I

The methods used are described in the previous article (Hermansen, Hultman and Saltin 1967) except for the blood glucose determination which, in this study was made by glucose oxidase method (Hjelm and de Verdier 1963) These experiments were performed the Department of Physiology Gymnastik och Idrottshogskolan. The sequence of the measurements is show Fig 1 The determination of the glycogen content in needle biopsy specimens from the lateral portion of the quadriceps femoris was made according to the method described by Hultman (1967 Muscle biopsy specimens were taken before exercise started, and immediately after the subjects were exhausted

The week schedule for both diet and work is illustrated at the top of Fig 1 The subjects were given mixed, uncontrolled diet prior the first measurements of muscle glycogen and work time The work consisted of pedalling bicycle to exhaustion at work load corresponding to an oxygen uptake of 3 15 (2 4—3 7) l min, which equals 75 1—83 per cent of the subjects maximal oxygen uptake On the day of the experiment, the subjects had no breakfast before the exercise test Six of the 9 subjects were then given fat — protein P diet for 3 days before the next work period The work again consisted of pedalling exhaustion the 5 per cent work load On the next 3 days the subjects were limited to predominantly carbohydrate C diet before the last work experiment The remaining three subjects also followed the aforementioned schedule except that they were first given the carbohydrate diet, followed by the fat — protein diet. All the food

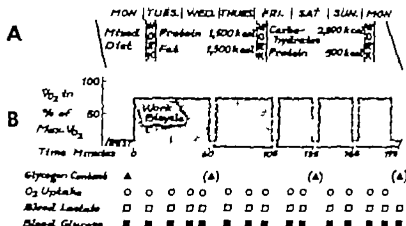


Fig 1 A. Week schedule for diet and work programme in 6 subjects. I 3 subjects the tw last diet periods were interchanged.
B. Schedule for the measurements in connection with the exercise test.
The second biopsy for glycogen determination (▲) was made immediately after the work period, which was of different length depending on the type of diet.

eaten by the subjects when they were on the controlled diet was prepared and served at the laboratory During work, the subjects drank w ter with some electrolytes, to minimize the effect of sweating For psychological reasons, 5-min est period was inserted in the cor tinuous exercise t fixed intervals (Fig. 1 b)

TABLE II Prolonged physical exercise after three periods of different diets (mixed = M, fat + pro- *carbohydrate (CH g) oxygen uptake (O₂ l/min)*

Subject	After M diet					After P diet	
	Muscle glycogen		W T min	Used CH g	O l/min	Muscle glycogen	
	Before	After				Before	After
T.P	2 51	0 07	113 4	413	3.86	1.39	0 08
B.T	1 19	0 35	148.5	375	2.99	0.60	0 32
R.S	1 63	0.10	121 5	266	2.94	0.42	0.39
A.L.	1 91	0.10	150.5	191	2.47	0.58	0 23
S.P	2 20	0 53	117 3	292	2.93	0 31	0 20
S-O J	2 11	0 06	124 5	423	3 67	0.91	0.10
Mean	1 93	0.20	125.8	327	3 14	0.69	0.19
C.F	1 32	0.11	94.5	279	3.61	0.48	0 03
R.E.	1 [illegible]0	0 16	84 7	210	3 11	0 48	0 00
K-G.G	1 [illegible]	0 01	88 0	306	3.55	0.56	0 02
Total mean	1	0 17	113 6	306.4	3 24	0.63	0 13
± S.E.	[illegible]1	0 05	± 5.3	± 27 4	±0.15	0 10	±0 05

I these 3 subjects the diet schedule was M, C, and P; not as in the 6 others (M P and C)

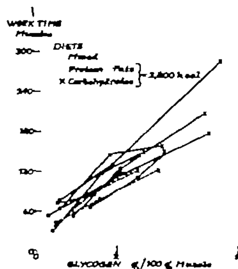


Fig 2. Relation bet een initial glycogen content in quadriceps femoris and work time. Equation for regression line: y = 41.6 + 36.8, = 0.92, $p < 0.001$ - - - the 3 subjects with carbohydrate diet prior to the fat + protein one.

Results

In Table II and Fig 2, the individual values are given for the *muscle glycogen content* in relation to the maximal work time on a work load corresponding to 75 per cent f the maximal oxygen uptake. A good correlation is present betw een the

teln = P carbohydrate = C) Muscle glycogen (g/100 g muscle) work time (W T min); used

			After C diet				
W T min	Used CH g	O_2 l/min	Muscle glycogen		W T min	Used CH g	O l/min
			Before	After			
68.5	159	3.86	3.18	0 27	150 0	544	3.75
66.6	88	3.12	3.11	0.53	160.6	429	3.02
30 3	54	2.76	2.66	0 60	150 3	329	2.80
73 3	71	2 41	4 68	0.30	283.0	574	2.44
56.1	88	3.00	4.31	0.56	180 0	533	3.06
36 0	67	3.41	4.24	0.43	210.0	798	3.54
38 8	88	3 09	3 70	0.43	189 3	533	3 10
41 6	77	3.70	2 48	0 59	123 8	348	3 61
43 5	71	3 03	3 00	0 53	119 0	360	2 92
3 0	91	3 22	2 10	0 07	120 0	416	3 30
36 9	83 1	3.17	3 31	0 43	166 3	481 5	3 16
±1 7	10 1	±0.13	±0 30	±0 06	17 8	4 1	±0 14

TABLE III Statistical treatment. mean values of data (Table II and Fig 3) obtained before, during, differences after the three diets (C, M, P)

C = carbohydrate diet
M = mixed diet
P = fat + protein diet

		n	C	M
			mean ± S.E.	mean ± S.E.
Muscle glycogen g/100 g	before work	9	3.31 ± 0.30	1.75 ± 0.13
	after work	9	0.43 ± 0.06	0.17 ± 0.03
Work time min		9	166.5 ± 17.8	113.6 ± 5.3
Utilized carbohydrat g	during work	9	481.5 ± 47.1	306.4 ± 27.4
Oxygen uptake l/min		9	3.16 ± 0.14	3.24 ± 0.15
Blood pyruvate mM/l	at rest	6	0.163 ± 0.011	0.120 ± 0.003
	after 30 min work	6	0.238 ± 0.023	0.237 ± 0.011
	at end of work	6	0.228 ± 0.038	0.179 ± 0.017
Blood lactate mM/l	at rest	6	1.73 ± 0.17	1.02 ± 0.14
	after 30 min work	6	4.92 ± 0.74	5.22 ± 0.91
	at end of work	6	3.61 ± 0.74	2.63 ± 0.53
Respiratory quotient	at rest	6	0.943 ± 0.024	0.815 ± 0.004
	after 30 min work	6	0.912 ± 0.009	0.915 ± 0.007
	at end of work	6	0.918 ± 0.012	0.882 ± 0.020
Blood glucose mg/100 ml	at rest	6	91.7 ± 5.9	76.8 ± 4.5
	after 45 min work	5	77.3 ± 3.4	76.3 ± 8.6
	at end of work	6	63.3 ± 2.1	53.8 ± 6.2

initial muscle glycogen concentration and the maximal work time over the whole range of initial glycogen values, as well as in each subject. The muscle glycogen averaged 1.75 0.63 and 3.31 g/100 g wet muscle after the M P and C diet, respectively. The mean maximal work times in the corresponding situations were 113 57 and 167 min, respectively. The mean decrease during exercise in muscle glycogen (mg/100 g tissue/min) was 14.2 ± 1.40 8.78 ± 1.70 and 17.4 ± 0.85 after diets M P and C. The differences in decrease M—P and C—M had p values of <0.0 and <0.0 respectively (paired t test).

The three subjects (C I R E. and K.-G G) given the C diet prior to the P one had markedly lower values for the muscle glycogen content after the C diet, compared to the other six subjects (cf Table II and Fig 2). Therefore only six subjects following the main procedure are included in Figs. 5 and 6, where a comparison is made between the average values for blood pyruvate blood lactate RQ blood glucose (Fig 3 heart rate and oxygen uptake (Fig 6) after the three diets. These values are also used for calculation of the statistics (cf the lower part of Table III).

The mean *blood pyruvate* at rest was 0.12 0.09 and 0.16 mmole/l after the M P and the C diet, respectively. During the first 30 min of exercise the pyruvate level

and after work following the three diets. Probability calculated on paired t test of intra-individual

P	C—M	C—P	M—P
mean ± S.E.	p	p	p
0.63 ± 0.10	<0.001	<0.001	<0.001
0.13 ± 0.05	<0.01	<0.01	>0.1
56.9 ± 1.7	<0.01	<0.001	<0.001
85.1 ± 10.1	<0.005	<0.001	<0.001
3.17 ± 0.15	>0.1	>0.1	>0.1
0.092 ± 0.008	<0.05	<0.01	<0.05
0.187 ± 0.017	>0.1	<0.05	<0.05
0.183 ± 0.020	>0.1	>0.1	>0.1
0.75 ± 0.17	<0.05	<0.01	>0.1
2.45 ± 0.18	>0.1	<0.01	<0.01
2.38 ± 0.24	<0.05	<0.05	>0.1
0.745 ± 0.029	<0.01	<0.01	<0.05
0.813 ± 0.009	<0.01	<0.01	<0.01
0.795 ± 0.014	<0.01	<0.01	<0.01
84.3 ± 4.0	>0.1	>0.1	>0.1
52.6 ± 2.6	>0.1	<0.05	<0.05
50.7 ± 10.8	>0.1	>0.1	>0.1

was significantly lower after the P diet than after the M and C diets (*cf* Table III and Fig 3)

The *blood lactate* at rest was 1 0 0 8 and 1 7 mmole/l (*cf* Fig 3) after the respective diets (M P and C) It increased during the first 30 min of exercise to 5.2, 2.5 and 4.9 mmoles/l. At the end of exercise immediately before exhaustion, the blood lactate concentration had fallen to 3 7 mmoles/l after the C diet, and to 2.7 mmoles/l after the M diet. The last reduction was significant. After the P diet, the blood lactate was essentially unchanged during exercise. Statistical treatment of intra individual differences after the three diets is given in Table III

The RQ at rest was 0.81 0.74 and 0.94 after the respective diets (cf Fig 3) It increased during the first 15 min of exercise to 0.93 0.84 and 0.97 respectively When the exercise proceeded, there was a slight reduction in the mean for the RQ with all diets but the decrease was not significant The probability of differences in RQ after the three diets is given in Table III

The mean *blood glucose* values are given in Table III and Fig 3 During exercise there was a fall in blood glucose from the beginning of exercise except after the M diet, when a transient increase was first noted. At 45 min exercise the concentrations after the C and the M diets were almost significantly higher than the cor

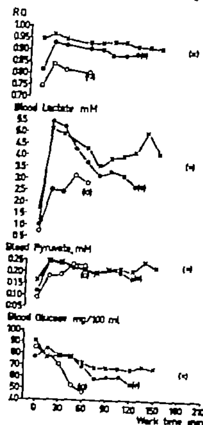


Fig 3. Mea lues of RQ, blood lactate, pyruv te and glucose in connexion ith exercise after different diets in 6 ubjects carbohydrat diet ● mixed diet, ○ fat + protei diet ( ) denotes the lue end of exercise

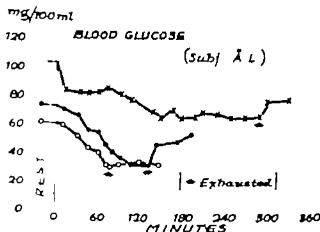


Fig. 4 Blood glucose onnexion h exer ise fter d fferen iets in subject Å.L. × carbohydra d ● l l fa pr diet

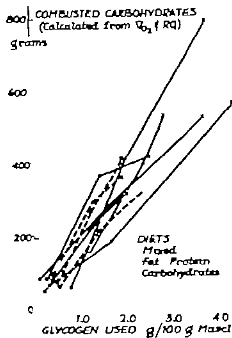


Fig 5.

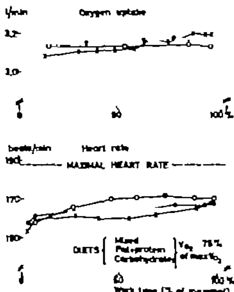


Fig. 6.

Fig 5 Relation between used muscle glycogen and total utilization of carbohydrates during exhaustive exercise Symbols as in Fig 2. Equation of regression line: y = 148.52 + 43 8, = 0.92, $p < 0.001$

Fig 6. Heart rat and oxygen uptake during exercise Mean alues in 6 subjects.

responding alues after the P diet (*cf* Table III) A slow recovery in the blood glucose concentration was marked in some ubjects after the P diet In subject Å.L. *f* Fig 4) the blood glucose concentration at exhaustion after the P d et was 52 mg/100 ml, and after 60 min recovery it was still only 34 mg/100 ml.

The *total amount of arbohdrate* combusted during the work period calculated from the oxygen uptake and RQ, is related to the amount of glycogen utilized, *i.e.* the difference between the muscle glycogen content before and after exercise (*f* Tables II and III Fig 5)

The calculated alues for tilized carbohydrates during exercise ranged from 54 g to 798 g In all instances there wa a good correlation to th glycogen eduction in the quadriceps, which ranged from 0 11 to 4 38 g/100 g wet muscle The mean decrease in muscle glycogen (mg 100 g tissue g used carboh drate) was 41 + 0 63 71 – 1 02 and 6 02 ± 0 33 after th respecti diets M P and C The differences between the groups were not ignificant

Fig 6 Illustrates the *heart ate* during exercise after the d fferent d ets Th differences were mall and not significant

The *oxygen uptake* during exercise was n the a erage 3 13 l min. egardless of

the diet preceding the experiment. Nor were any significant changes noted in a comparison between the beginning and end of exercise.

The three subjects given the carbohydrate diet before the fat + protein diet showed similar results to those presented for the six subjects in Figs. 3 and 6, except that the differences between the M and C diet were smaller.

Discussion

The results of the present experiments demonstrate that the muscle glycogen concentration can be varied within a wide range, provided that different diets are administered after exhaustive exercise causing depletion of the local muscle glycogen stores. This is in agreement with previous studies (Bergström and Hultman 1966 b, Hultman and Bergström 1967). Thus, three days of fat + protein diet after exhaustive work did not resynthetize the muscle glycogen content to more than about 50 per cent of the initial value, whereas the carbohydrate diet raised the concentration to far above the normal range (0.95—2.0 g/100 g muscle) (Hultman 1967). Three subjects reached values above 4 g/100 g muscle, which are the highest ever reported in healthy males. It should be recalled that the three subjects given a C diet after the M diet did not reach as high muscle glycogen values as the 6 subjects given the P diet before the C one. Thus a period of carbohydrate-free diet (*i.e.*, a period with low muscle glycogen) seems to further stimulate glycogen synthesis when carbohydrates are given (Ahlborg *et al.* 1967 b, Saltin and Hermansen 1967). It should be pointed out that the effect of the M diet on the muscle glycogen formation is not comparable to that of the P and C diet, as the M diet period was not preceded by glycogen-depleting exercise.

It is well established that the capacity for exercise is directly dependent on the individual's maximal oxygen uptake (Åstrand 1956). To minimize the inter-individual variation in work time during prolonged exercise, the work load for each subject was selected so that it represented about 75 per cent of his maximal oxygen uptake. All the subjects were somewhat trained (*cf.* maximal oxygen uptake in Table I), which implies that the exercise performed during the first part of the study should not have improved their performance in the last experiment. In subject Å.L., who had the lowest maximal oxygen uptake, measurements of his maximal uptake before and after the experimental period showed identical values (3.35 and 3.36 l/min).

The good correlation between initial glycogen concentration and work time (Fig. 2) demonstrates that the individual's ability to sustain prolonged exercise is highly dependent on the glycogen content of the muscles which, in turn, is dependent on the type of diet before exercise. It is improbable that the degree of fitness plays an essential role either in the variation in muscle glycogen concentration or in its importance for the performance capacity since the best trained subject (S.-O. J.) and the least trained one (Å.L.) behaved similarly. These two subjects had the most marked increase in muscle glycogen concentration after the C diet, and they could also perform the longest on the 75 per cent work load.

Christensen and Hansen (1939) have earlier shown that the ability to perform prolonged exercise on a given work load is dependent on the type of diet before exercise. They concluded that carbohydrates were the most essential fuel during heavy muscular work. The results of the present study fully confirm this conclusion. It is of special interest that the performance time on a given load can be increased by more than 100 per cent by instituting a carbohydrate-rich diet after exhaustive exercise, and that the muscle glycogen concentration seems to be the key factor for the increase in performance capacity for prolonged work. This method of increasing the performance capacity may have practical applications in such situations as manual labour military activities and athletics.

The output of glucose from the liver during prolonged exercise has been found to amount to 300 mg/min (Rowell, Masoro and Spencer 1965) At the end of prolonged severe exercise a marked further increase in the glucose output was observed (Hultman, to be published) The blood glucose concentration during and after exercise fell to extremely low levels only after the P diet. In this situation the subjects experienced fatigue, not only localized to the legs but also generally Some subjects suffered from headache and dizziness. The low blood sugar values at the end of exercise and the slow increase in blood sugar after exercise, especially after the P diet, might indicate a relative depletion of the glycogen stores in the liver in this situation.

It has been shown earlier that the diet can influence the relative role of fat and carbohydrate as fuel at rest and during exercise (Christensen and Hansen 1939) This was also manifested in the present study by a significantly higher RQ both at rest and during exercise after the M and C diets on the one hand, than after the P diet on the other hand. Furthermore the calculated consumption of glycogen per time unit is lower after the P diet than after M and C diets. After the C diet both the RQ and the glycogen consumption per time unit were highest. This is in accordance with the observation that the regression line between performance time and initial muscle glycogen does not pass through zero (Fig 2)

Blood lactate and pyruvate levels at rest and during the first part of exercise were also significantly lower after the P diet. These data suggest that the muscle cells have an ability to adapt to oxidizing more fat also at extremely high work loads, provided that the carbohydrate supply is low during the days before exercise. In contrast to this, the constantly high RQ throughout the period of prolonged heavy exercise after the carbohydrate and mixed diets indicates that the carbohydrate supply must in fact, be limited for some time before exercise to permit the adaptation to fat combustion to take place

As illustrated in Fig 5 a good correlation is present between the glycogen used in the working muscles and the amount of carbohydrate utilized calculated from the oxygen uptake and RQ This applies over a wide range of RQ values and muscle glycogen concentrations. The glycogen decrease in relation to total carbohydrate consumption was not significantly different in the three diet groups This indicates that the muscle glycogen store is the most important carbohydrate source during

heavy exercise. After the C diet, some subjects had available carbohydrate stores up to 700—800 g, i.e. almost twice the figure presented by Hedman (1957) during cross-country skiing. His values are, however, compatible with those obtained by us after the M diet. Assuming that 20 kg of muscle are involved in the bicycle exercise, the exceedingly high figures after the carbohydrate diet noted in the present study are reasonable since the reduction in glycogen concentration in the quadriceps femoris muscle during exercise was up to 4 g/100 g muscle.

The higher muscle glycogen concentration at exhaustion after the C diet may indicate that other factors ultimately limit the performance in this situation. Although psychological factors may have been of importance, it cannot be concluded that this is the only explanation.

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Acta physiol. scand. 1967 71 151—162

From the Department of Physiology Karolinska Institutet Stockholm, Sweden

Reuptake and Net Uptake of Noradrenaline in Adrenergic Nerve Granules with a Note on the Affinity for l and d Isomers

By

U S von Euler and F Lishajko

Received 3 April 1967

Abstract

Euler, U S v and F Lishajko. *Reuptake and net uptake of noradrenaline in adrenergic nerve granules with note on the affinity for l- and d-isomers* Acta physiol. scand 1967 *71* 151—162

The net loss of noradrenaline (NA) from bovine splenic nerve granules observed after incubation for 60 min 20 C in phosphate buffer is reduced in the presence of NA in the incubation medium. The effect is observed from 10 M NA and gradually increases so tha at 10 M NA in the medium the net loss is small By continuous removal of he NA in the medium by addition of potassium ferricyanide the net loss reaches maximum alue which may represent the basic release rate The effect of NA in the medium is due t reuptake of NA as indicated by the incorporation of dl-H NA in the granules during incubation. Preliminary observations suggest that the reuptake is lso ATP-dependent During successive depletion of labelled stores at 20 the specific activity of NA is unaltered indicating uniformly labelled pool. I partially NA depleted nerve granules the net uptake of NA from the medium is enhanced by ATP Mg and dependent on the NA concentration in the medium. A method for estimating the relativ affinity of the l- and d-isomer for nerv granules is described. The mean value found for the affinity ratio l-NA/d-NA was 9 4 (range 4 2 —15.7) I is suggested tha the continuous turnover of NA in the nerv storage granules is of major significance for the physiological transmitter homeostasis.

Since the demonstration of specific noradrenaline (NA) storage particles in the high speed sediment of homogenates of adrenergic nerves and of organs supplied with such nerves (Euler and Hillarp 1956 Euler 1958) a large number of studies have been made on these organelles indicating thei bility to release take up and store the ad energic transmitter and some related amines (cf Potter and Axelrod 1963 Westfall 1965) The spontaneous NA release rate from a uspension of nerv granules is high (Euler and Lishajko 1963 a) compared w th that f adrenal medullary gra ules Hillarp and Nilson 1954) The uptake of NA in nerve granules is greatly enhanced by ATP Mg (Euler and Lishajko 1963 b) Certain observations indicated that the uptake process l of twofold nature specific uptake t low NA concentrations which is inhibited by reserpine and an "unspecific uptake t higher concentrations,

about 10^{-4} M, which is reserpine-resistant (Euler and Lishajko 1963 c). A reserpine resistant NA uptake in mouse heart granules has been recently reported by Stitzel and Lundborg (1967).

In the presence of NA in a concentration of 0.5—1 × 10^{-4} M the net loss of NA on incubation of granules in isotonic phosphate buffer pH 7.5 is greatly retarded (Euler and Lishajko 1963 a). This effect is due to an uptake of NA concomitantly with the release (Euler, Stjärne and Lishajko 1963).

In the present paper we wish to report some observations on the spontaneous NA *release and reuptake of NA in isolated adrenergic nerve granules. The significance* of the reuptake in nerve granules is discussed.

Methods

Preparation of granules. Bovine splenic nerves were collected at the slaughter house and brought to the laboratory on ice. After dissection and desheathing, the nerves were cut in pieces and homogenized in an Ultra-Turrax apparatus (Janke & Kunkel, Freiburg) at 13,500 rpm for 30 sec, using about 10 ml ice-cold 0.13 M potassium phosphate buffer of pH 7.5 per g of nerve. The homogenate was centrifuged for 10 min at 9 000 × g and the supernatant, containing the NA granules, used for incubation. The NA concentration in the granule suspension under these conditions is 1—3 × 10^{-6} M. The suspension, 8 ml per tube, was incubated in a water bath, without stirring, at 20 or 37° for various periods of time with addition of NA and ATP as indicated in the experiments. As a rule $MgCl_2$ was not added to the original granule suspension since this contained Mg^{++} in optimal concentrations, but after resuspension Mg^{++} had to be added in order to permit ATP-action. dl-7-^{3}H NA (New England Nuclear Corp., s. a. 5 C/mmole, chromatographically purified) was added in a concentration of 2—5 × 10^{-8} M which did not noticeably alter the net release rate and could be regarded as a tracer concentration. After the incubation the suspension was centrifuged at 50,000 × g for 30 min and the pellet extracted with 0.4 M perchloric acid. If NA or some other amine had been added to a concentration of 10 or higher the pellet was resuspended in fresh phosphate buffer after centrifugation and sedimented a second time before extraction and assay. Aliquots were used for fluorimetric estimation of NA (Euler and Lishajko 1961) and for measuring radioactivity (Stjärne and Euler 1965). No correction was made for the small amounts of NA and radioactivity in the wash fluid, usually 2—3 per cent of the total amount in the supernatant.

The specific activity of NA in the supernatant was measured after adsorption of the NA on an alumina column and elution with 0.25 N acetic acid.

The proportion of incorporated radioactive NA was expressed as the ratio of the specific activity of the sediment (SA_{sed}) and the supernatant (SA_s) respectively at the end of incubation.

Absolute release rate may be expressed as the percentage loss per min (k) calculated from the percentage (p) of the original amount of NA, remaining after incubation for t min according to the equation p = 100 e^{-kt}. Relative release rate is expressed as $\frac{2-\log p_E}{2-\log p_C}$ based on the percentage remaining NA values in the sediment after incubation in the control (p_C) and in the experiment (p_E) at a given time. Half-time (t/2) = $\frac{\ln 2}{k} = \frac{t \times \log 2}{2-\log p}$ using the same symbols as above.

Results

1. *Effect of NA concentration in the medium on NA release rate from granules at 20° and 37°.*

In the standard procedure used in most experiments the mean NA release rate at 20° from a suspension of nerve granules containing endogenous NA in a concentration of 1—3 × 10^{-6} M was determined to 0.94 per cent per min (t/2 = 74 min, p = 57 ± 3.5 (S.D.), n = 37). At 37° the release rate was about 10 times higher or 9.8 per cent per min (t/2 = 6.8 min, p = 36 ± 4.8 (S.D.), n = 23).

The previously demonstrated retardation of the net NA release from granules

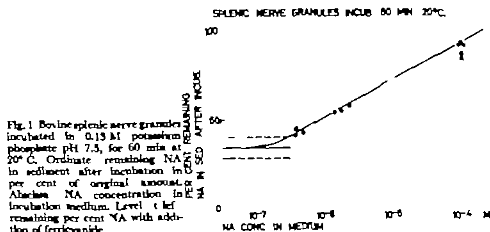


Fig. 1 Bovine splenic nerve granules incubated in 0.13 M potassium phosphate pH 7.5, for 60 min at 20° C. Ordinate remaining NA in sediment after incubation in per cent of original amount. Abscissa NA concentration in incubation medium. Level [illegible] left remaining per cent NA with addition of ferricyanide

Table I Values of release constant (k) at different NA concentrations in incubation medium (NA loss per min) at 20

Molar concentration NA	k × 10[illegible]
0	1.8
3 10[illegible]	1.5
10[illegible]	1 1
3 10[illegible]	0.80
10[illegible]	0.55
10[illegible]	0 12

incubated with NA 0.5—1 × 10^{-4} M (Euler and Lishajko 1963 a) was confirmed and the NA release rate determined for different concentrations of NA in the incubation medium. The percentage remaining NA in the granules after standard incubation 60 min at 20 C with different NA concentration in the medium is illustrated in Fig. 1 The values for the "release" constant k (relative NA loss per min) at different NA concentrations in the medium are given in Table 1

In order to study the effect of lower NA concentrations the granules were sedimented and resuspended in fresh phosphate buffer Since after resuspension the NA concentration in the medium gradually increases during incubation, the NA concentration has been taken as the mean of the pre and postincubation values. The preincubation NA value has been 1—1 5 [illegible] 10 [illegible] M It was consistently noted that an increase of the NA concentration in the medium by addition of NA, from 1 3 10 [illegible] M to twice this concentration, or from 5 to 50 ng per ml increased the percentage remaining NA in the granules after incubation.

As resuspension fluid 0 13 M potassium phosphate at pH [illegible] was used In some experiments the effect of addition of $MgCl_2$ 3 mM $CaCl_2$ 1 mM and N[illegible] Cl 3 mM

TABLE II. Effect of potassium ferricyanide on remaining NA in splenic nerve granules after incubation 60 min, 20 C in the presence of some drugs

Added	Conc. M	Per cent remaining NA in sediment Control (unincub. = 100)	$K_3F(CN)_6$ 1—5 × 10^{-4}M
No addition	—	60	34
ATP	3 × 10^{-4}	93	39
ADP	3 × 10^{-4}	81	39
Prenylamine	3 × 10^{-4}	6.9	2.3
Reserpine	10^{-4}	91	82
Phenylethylamine	10^{-}	18	16

TABLE III. Effect of potassium ferricyanide added to the incubation medium on the relative release rate of NA from splenic nerve granules (control = 1)

$K_3F(CN)_6$ concentration	3 × 10^{-4}	10^{-4}	2 × 10^{-}	5 × 10^{-4}	8 × 10^{-4}	1.6 × 10^{-3}M
Release rate M ± SEM	1.18	2.01 ± 0.13	2.01 ± 0.18	1.94 ± 0.06	1.82 ± 0.08	1.97
Number of expts	(1)	(6)	(5)	(11)	(8)	(1)

as tested. In the presence of Na and Mg^{++} added together the remaining NA in the sediment after incubation was increased by 5—10 per cent. Addition of Ca^{++} caused a slight decrease of the reuptake.

2. NA release in absence of NA in the medium

In order to study what may be termed the "basic" release rate we have attempted to exclude the effect of uptake by continuous removal of the NA in the medium. Addition of aluminium oxide to the incubation medium has previously been used for this purpose but preliminary tests showed that this procedure was less suitable for the present experiments. We have therefore added an oxydant, potassium ferricyanide, to the incubation medium. This compound in concentrations from 3 × 10^{-} to 1.6 × 10^{-3} M appeared to have only little, if any, deteriorating effect on the granules, since their ability to retain and take up NA after removal of the Fe^{+} by ascorbic acid did not seem to differ from that of normal granules. Moreover the strong NA-releasing effect of prenylamine and phenylethylamine or the retarding effect of reserpine on the granules were not prevented by ferricyanide (Table II).

At a ferricyanide concentration of 10^{-} M a marked increase in NA release rate was noted. This effect was not consistently increased by raising the ferricyanide concentration up to 1.6 × 10^{-} M. In the majority of the experiments the concentra-

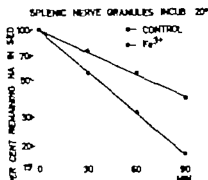


Fig 2 Splenic nerv granules, incubated t 20 C as in Fig 1 Open circles control, filled circles incubation in presence of 10^{-4} M potassium ferricyanide Ordinat per cent remaining NA in sediment after incubation.

tion 5×10^{-4} M was used (1 65 mg/ml) Tabl III shows the effect of different concentrations of potassium ferricyanide in the incubation med um on the relat e release rate of NA (control = 1) The constant value of the increased release rate is also suggestive evidence that the ferricyanide does not cause any damage to the granules over a wide concentration range

As seen in Table II ferricyanide prevents most of the retarding effects of ATP or ADP on the NA release, which is n agreement with the finding that this effect is due to concomitant uptake The small excess of NA in the ATP and ADP experi ments may ndicate an inhibitory action of the nucleotides on the effective NA release or could be due to a rapid reuptake aided by exogenous ATP or ADP

The effect of continuous NA removal is illustrated in Fig 2 wh ch show the effect of ferricyanide on the release of NA from the granules at 20 (mean of 2 expts.) The difference between the two curves is regarded as representing the amount of NA taken up during incubation in a medium containing NA 1—3 × 10 M.

A number of other oxidants ha e been tested on the NA release rate such as iodine and various heavy metal salts. All of the compounds tested caused, however a trong release uggesting damage to the granules or inactivation of bound NA.

5 *Effect of NA concentration in the medium and f ATP on incorporation of dl-³H NA*

The incorporation of radioactive NA in the granules wa determined d rin g incuba tion for 60 min at 20 C and expressed as the specific activity ratio of NA in sediment and supernatant after incubation (SA_{SED}/SA_{sup}) In the presence of NA in a concentra tion of 1—3 × 10^{-4} M in the incorporation of labelled NA was followed at time

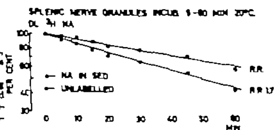


Fig 3 Bovine splenic nerv granules, incuba ed at 20 C as in Fig 1 Endogenous NA 3 10 M Added dl-³H NA 10 M Open circles per cen remaining NA in sediment after incubation Filled circles nonlabelled portion of same sediment Ordinate remaining NA in sediment. R R — relativ release rate

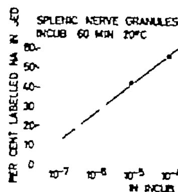


Fig 4 Splenic nerve granules, incubated at 20 C as in Fig 1 Ordinate incorporated portion of labelled NA after incubation. Abscissa NA concentration in incubation medium.

intervals up to 60 min as illustrated in Fig 3 From the figure it can be seen that incorporation of labelled NA represents the difference between two exponential courses reaching about 90 per cent of its maximal absolute value in 60 min. In a series of experiments the incorporation of dl- H NA during 60 min incubation at 20 in the presence of NA 1—3 10⁻⁴ M amounted to 30 ± 4.8 (S.D.) ($n = 17$) per cent of the remaining NA, corresponding to a relative release rate of 1 7 for the nonlabelled portion.

The uptake of nonlabelled and labelled NA was also measured on addition of NA in concentrations 10 M and 10⁻⁴ M and in resuspended granules, with an average NA concentration in the medium of less than 10⁻⁴ M. The addition of NA in concentrations 10⁻⁴ M and 10⁻ M had a strong retarding effect on the net release of NA owing to concomitant uptake. As seen in Fig 4 the proportion of labelled NA expressed by the SA_{sed}/SA_{inc} ratio increases with rising concentrations of NA in the medium. At low concentrations of NA in the medium, as after sedimentation and resuspension, the incorporation of labelled NA was small, about 20 per cent of the remaining NA.

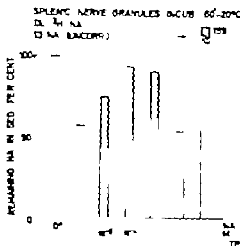


Fig 5 Splenic nerve granules, incubated 20 C as in Fig 1 NA in medium —3 10⁻⁴ M Added to each tube 5 10⁻⁵ M dl- H NA and NA, ATP as indicated. Columns represent remaining NA in sediment after incubation in per cent of original amount. Stippled area labelled NA, calculated from SA_{sed}-SA_{inc}-values

Addition of ATP in a concentration of 3—4 $\times$ 10^{-4} M to the incubation medium causes a very marked retardation of the NA release owing to uptake of NA as previously observed. This extra uptake is considerable even at a NA concentration of 1—3 $\times$ 10^{-6} M and is further increased at NA concentrations of 10^{-5} and 10^{-4} M (Fig 5)

The proportion of labelled NA in the sediment in the presence of ATP showed a similar dependence on the NA concentration in the incubation medium as in the experiments without ATP (Fig 5) The higher remaining amount of nonlabelled NA after incubation in the presence of ATP may indicate some inhibition of release or possibly rapid reuptake with the aid of exogenous ATP

4 *Net uptake of NA after partial depletion of granules*

After 10 min incubation at 37° the remaining NA was about 35 per cent of the original amount. As shown previously (Euler ud Lishajko 1963 b) continued incubation for 30 min at 20 C resulted in a further release occurring at approximately the same rate as from the original amount when measured at 20° C. In the presence of NA 10^{-4} M a small net uptake is sometimes seen. Addition of NA 10^{-5} M usually reduces the NA loss during the second incubation but does not cause a net uptake. Addition of ATP however causes a net uptake of NA, provided that NA is present in the incubation medium. Thus at a concentration of 1—3 $\times$ 10^{-6} M the net uptake is considerable (Fig 6) Further addition of NA usually increases the net uptake so that at the end of incubation the granules have regained a considerable part of the NA lost during the first incubation.

In this kind of experiment the incorporation of labelled NA was studied in a similar way as in the release experiments. Addition of label before the second incubation period caused an uptake of radioactive NA corresponding to about one third of the remaining NA in the controls. The proportion of labelled NA was again higher when NA was added, more after 10^{-4} M than after 10^{-5} M. Particularly after addition of

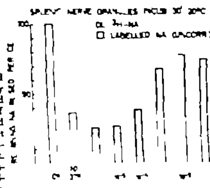


Fig 6 Splenic nerve granules, incubated at 20 C as in Fig. 1 Partial depletion by preincubation 10 min at 37 C, continued incubation 30 min 20 C. Addition of 3 10 M dl-H NA to each tube and NA, ATP as indicated. Columns represent remaining NA in sediment after incubation, in per cent of original amount. NA concentrations in medium before addition 1—3 10^{-} M. Stippled area labelled NA calculated from S.Vego/ΣA ratio

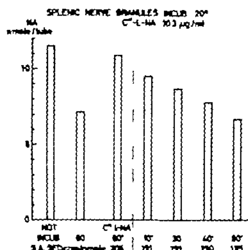


Fig. 7 Splenic nerv granules incubated at 20 C as in Fig. 1 Col 3 incubated with l-14C-NA 60 min After sedimentation and resuspension in phosphat buffer repeated once incubation t 20 for 10—80 mi (ol. 4—7). Bottom row spec. act. of NA i sediment, cpm/nmole

NA 10 M the uptake of labelled NA is high, indicating that NA facilitates the release or exchange of endogenous NA when present in high concentrations under the prevailing experimental conditions As in Fig 5 the proportion of nonlabelled NA is higher when ATP has been added.

3 *Rate of release of radioactive NA incorporated in granules*

Nerve granules were incubated with l C-NA (kindly given to us by Dr I Kopin, Bethesda) n a concentration of 6 × 10⁻ M for 60 min at 20 C After repeated sedimentation and resuspension in fresh phosphate buffer the granules were incubated for 10—80 m n at 20 C At the end of the incubation period the granules were sedimented and radioactivity and NA content determined

A seen n F g 7 the pecific activity of the NA remaining in the granules after n ubatio n maintained a onstant value indicating that the radioactive NA incorporated n th gr nules was released at the same rate a the endogenous NA.

4 *Note on the aff ty quotie t for the uptake of l and d NA in isolated granules*

If the aff uty quo tient for the uptake of l and d NA in isolated nerve granules is greater than un ty th hould lead to a smaller uptake of radioactive dl-NA in the granules than f ffin ties wer equal provided the specific activ ty of the d-isomer is lower than that of th l somer in the incubation medium. From the deficit and the specific activ t es d l nd d N A t possible to calculate the elative affinity for the uptake of the isom

If the affinity atio l NA d NA is called A, and Q represents the concentration ratio for d and l NA n th n ubation medium, the amounts of d and l-NA taken

up are $\frac{Q}{Q+A}$ and $\frac{A}{Q+A}$ espe tiv ly Since the relativ specific activities are for

d-NA $= \frac{1}{Q}$ and for l-NA $= 1$ the number of counts for d-NA $= \frac{1}{Q+A}$ and for l-NA $= \frac{A}{Q+A}$. The sum $\frac{1+A}{Q+A}$ then represents the relative number of counts in the sediment. If the value of Q exceeds or falls below unity the uptake differs from that at Q = 1 and reaches a maximum when Q approaches zero. At an l-NA/d-NA affinity ratio of e.g. 6 the maximum is 117 per cent of the value found at Q = 1.

An example is given below of actual estimation of the affinity ratio according to the principle outlined.

Isolated bovine splenic nerve granules were incubated in phosphate buffer pH 7.5 for 60 min at 20° C. To each tube was added dl-³H-NA to 10⁻⁶ M. d-NA added as indicated. Radioactivity was measured in sediment (SED) and the amounts of NA in supernatant (SU) and sediment determined (Table IV). From the table it can be seen that the average value for the affinity ratio is 9.2 in this experiment.

In two other experiments the similarly estimated affinity ratios were 9.6 (range 6.1—11.3, n = 4) and 9.5 (range 4.2—16, n = 5) the Q-values varying between 0.07 and 7.4. It should be pointed out that at Q-values below 1 the affinity values become slightly too high and at Q-values above 1 somewhat too low, owing to the shift in Q-values during the release of NA.

Estimations of l-NA/d-NA affinity ratios have been made previously by Iversen (1963), Maickel, Beaven and Brodie (1963) and by Stjärne and Euler (1965). The values obtained were 5.2, 11 and 5.9 respectively. The present results are in reasonably good agreement with those previously found based on other methods of estimation. While thus several reports agree as to a considerable preference for the uptake of l-NA into the stores it is worth noticing that no difference in release rate was observed for endogenous l-NA and incorporated d-NA in granules subjected to incubation at 20° C after loading.

TABLE IV. Estimated affinity ratio for binding of l-NA and d-NA to nerve granules, based on radioactive NA in sediment after incubation for 60 min at 20° and on concentration ratio d-NA and l-NA in the medium (Q)

Table number	cpm/sed 10⁻	NA/sed μg	added d-NA/ml	Q calc.¹	cpm/sed relative to value at Q = 1	affinity quotient A
3	25.6	1.2	0 10⁻⁴M	0.19	1.09	8.8
4	24.0	1.2	2 10⁻ M	0.67	1.0	15.7
	20.0	1.2	10 M	2.2	0.8	3.8
	17.1	1.3	1.2 10 M	4.1	0.3	4
	14	1.4	1.8 10 M	3	0.2	

¹ Calculated from recovery

Discussion

The results of the experiments with addition of ferricyanide to the incubation medium suggest that the presence of NA even in a concentration of 10^{-8} M is sufficient to maintain a degree of NA uptake which lowers the apparent release rate to about one half of the "basic" value, *i. e.* when no reuptake takes place. A certain reservation must be made for the possibility that ferricyanide does not wholly prevent reuptake. However, since an increase in ferricyanide concentration by 8 times does not increase the effect, we are inclined to believe that the effect is close to maximal.

The NA release rates for the nonlabelled portions in Fig. 3 and 5 suggest that the labelled part is closely related to the postulated reuptake. The difference between these values and the release rate observed with ferricyanide is too large to be neglected, but it should be taken into account that part of the nonlabelled NA released from the granules presumably will be recaptured as such in the absence of ferricyanide.

Since the effect of ATP is also largely prevented by addition of ferricyanide it may be assumed that this effect is mainly due to reuptake of NA, as also indicated by the incorporation of labelled NA.

If the release rate observed in the presence of ferricyanide is taken to represent the basic rate, it follows that higher relative release rates are due to a releasing action on the granular NA. This has been observed for tyramine, phenylethylamine, amphetamine and methamphetamine and several other drugs (Euler and Lishajko 1965 a, 1967). These results suggest that increased net release may be due to either one or both of two phenomena, inhibition of reuptake and direct release.

As observed previously (Stjärne 1964) the nonlabelled portion of NA remaining in the granules after incubation was increased in the presence of ATP. This observation suggests that during ATP-dependent reuptake the turnover of "exchangeable" NA molecules from the granules is to some extent inhibited. A reasonable explanation seems to be that the NA leaving or about to leave the granules is more rapidly or efficiently reincorporated in the stores, thus having less chances to become replaced by labelled NA.

As to the mechanism for the reuptake of NA into the granules from an incubation medium containing low or moderate concentrations of NA ($3 \times 10^{-}$—10^{-8} M) no definite statement can be made. In view of the enhancing effect of addition of ATP+Mg^{++} on the reuptake in undepleted granules and on the uptake of NA in partially depleted granules, it does not appear inconceivable that the spontaneous reuptake is ATP-Mg-dependent. Some indirect support for this assumption is found in the fact that the granules contain ATP (Schümann 1958, Stjärne 1964). Moreover, the results of preliminary experiments seem to indicate that factors which are known to inhibit ATP-action and uncouple oxidative phosphorylation such as dinitrophenol and other dinitrocompounds also prevent the reuptake of NA.

Reuptake into the nerve endings following release of NA, first suggested by McMillan (1959) is considered as an important means of inactivation of the neuro-

hormone (Whitby, Axelrod and Weil-Malherbe 1961). Consequently, inhibition of reuptake at the axon membrane may reinforce the action of exogenous NA and also increase the overflow of NA released on stimulation (Blakeley, Brown and Geffen 1964). The mechanism of the amine transport mediated by the "cell membrane pump" has recently been discussed by Carlsson and Waldeck (1965).

The results presented in this paper, partly in confirmation of earlier findings from this laboratory, indicate that a continuous reuptake of NA occurs at the granular level. The incorporation of labelled NA in vivo is in harmony with this concept.

Under resting conditions the reuptake of NA in the granules may play a significant role in the homeostasis of the neurotransmitter. A disturbance of this function would be expected to cause considerable alterations in net release. Support for this assumption is provided by the action of certain drugs. As previously shown (Euler and Lishajko 1963 b) reserpine effectively blocks the ATP-dependent net uptake of NA in partially depleted nerve granules and inhibits the reuptake of NA (Euler, Stjärne and Lishajko 1963). On the other hand reserpine does not seem to prevent uptake or reuptake through the axon membrane (Hillarp and Malmfors 1964). Since reserpine is a potent depleter of the NA stores, it follows that the inhibition of reuptake in the granules is the most important factor for the depleting effect of this agent, in spite of the fact that NA release rate from the granules is subnormal. Cocaine which is known to inhibit NA uptake in tissue (Whitby, Hertting and Axelrod 1960) and prevents the action of tyramine, but affects the granules only to a small degree in reasonable concentrations (Euler and Lishajko 1965 b), has only a weak or no depleting action (Muscholl 1961). It may therefore be that the events at the granular level are of greater significance for the overall action of a drug than the action at the axon membrane in this respect.

As shown in a recent publication (Euler and Lishajko 1967) tyramine acts partly by a direct NA releasing effect on the granules and partly by inhibition of reuptake in the granules. In high concentrations this amine — like some other indirectly acting amines — almost completely blocks reuptake into the granules and may in this way cause depletion of the stores, since the directly releasing effect is weak or absent at high concentration. Concomitant inhibition of NA reuptake at the axon membrane would presumably enhance this effect unless tyramine uptake is also inhibited.

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Acta physiol. scand. 1967. 71. 163—167

From the Institute of Occupational Health, Oslo, Norway

^{133}Xenon-Radiospirometry in Rabbits with Some Observations on Unilateral Hypoxia

By

Knut Dale

Received 6 April 1967

Abstract

Dale, K. *^{133}Xenon-radiospirometry in rabbits with some observations on unilateral hypoxia.* Acta physiol. scand. 1967. *71*. 163—167.

The application in the rabbit of [illegible] radiospirometric technique using ^{133}Xenon and external detectors is described. The ventilation and the perfusion could be measured separately for each lung. In unilateral hypoxia the method revealed [illegible] reduced perfusion of the hypoxic lung.

Small animals such as rabbits can be very useful in various types of studies within pulmonary physiology or pathology. It would be a great advantage for many types of pulmonary investigations in [illegible] rabbits if a technically simple and exact method was available for testing the function of each lung separately. Bronchospirometry is not easy to carry out in rabbits, and the method seems to be relatively time consuming (Dirken and Heemstra 1948).

^{133}Xenon-radiospirometry, first described by Knipping *et al.* in 1955, can replace bronchospirometry in man (Mormer 1967). The purpose of the present investigation was to see if a technique of radiospirometry with ^{133}Xenon was applicable in the rabbit, and if it could be used for analysis of the distribution of pulmonary blood flow in unilateral hypoxia.

Methods

The [illegible] shown in Fig. 1. The animal was placed on a table underneath which the two detectors were placed. An 0.5 cm thick lead plate covered with wood, which had holes for the lung fields cut after an X-ray picture, could be placed on the table with each hole exactly above the detectors. The NaI crystal used measured [illegible] inches in diameter and had the same thickness. A cylindrical lead collimator [illegible] 5 cm thick was used with the crystal recessed 3 cm and with the aperture touching the [illegible] The dual channel Picker scaler 600 [illegible] 045 which was calibrated before each experiment,

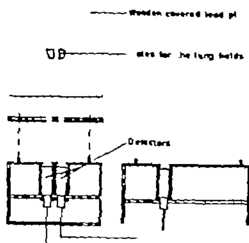


Fig 1 Schematical drawing of the experimental arrangement. The loose supporting plate on to which the animals were placed with their back down, is shown from above and in a cross-section. The animals' position was so arranged that its lungs were placed exactly above the holes for the lung fields which had been cut in the supporting plate. The plate which had a wooden layer and a lead layer (0.5 cm) was again placed and secured in the correct position above the detectors by screws which penetrated the two small holes shown. For further details, see text.

was used with a time constant of 0.1 sec and had a maximum counting rate of 300,000 cpm. The ratemeter had no pulse height analyser. The output of the ratemeter was linearly recorded on a two channel recorder.

To test the ability of the system to separate the activity from the two pulmonary fields, a pair of isolated lungs from a rabbit was separately inflated with air through each main bronchus. On the one side some ^{133}Xe was added to the air. When this lung preparation was examined on the table, less than 5 per cent of the total activity was recorded on the pure air side.

The rabbits were anaesthetized with an i.v. injection of 36 mg/kg of pentobarbitone (Nembutal® Abbott) and small supplementary doses were given when necessary. The animals were also given an intravenous injection of 0.05 mg/kg of atropine sulphate. Then a polyethylene catheter no. 80 was placed in the saphenous vein with the tip in the vena cava inferior. The rabbits were laid on their back on the lead plate and their position was arranged by fluoroscopy so that their lungs were exactly above the holes in the lead plate. The animals were then carefully attached to the plate which was again placed on the table as described. The ^{133}Xe was obtained from Amersham, England, in saline solution and with an initial concentration of 1.0 mC/ml. About 150 μC ^{133}Xe was injected through the catheter in the course of 1 sec.

The perfusion of each lung was considered directly related to the maximum counting rate H_{max} (Fig. 2; Heckscher, André-Larsen and Larsen 1966). The perfusion of each lung was expressed in per cent of the total perfusion (right + left lung). Because ventilation is the main factor causing disappearance of the radioactivity from the counting field, the *ventilation* of each lung could be calculated from the ^{133}Xe-wash-out rate (Kety 1951, Ball *et al.* 1962, Dollery *et al.* 1962). In man the clearance curve of the ^{133}Xe has been found to be multiexponential reflecting inhomogeneity of alveolar ventilation. In the present studies in the rabbits the initial steepest part of the wash-out curve was often apparently monoexponential but in some cases multiexponential. When initial concentrations of the ^{133}Xe is considered to be the same in all regions of the lungs, it is possible to calculate the average alveolar ventilation per unit alveolar volume from the formula used by Heckscher *et al.* (1966) for the relative initial slope of the wash-out curve:

$\dot{V}/\bar{V} = -C'(O)/C(O)$ ml/min/ml where $\dot{V}$ is the ventilation, $\bar{V}$ is the volume of the lung recorded from, C is the exponential function describing the initial part of the wash-out curve as a function of the time, $C' = \frac{dc}{dt}$ and C(O) is the maximal concentration of radioactivity in the lung at time zero, C(O) = H_{max} (Fig. 2). The relative initial slope C'(O)/C(O) is calculated from the following formula:

$\dot{V}/\bar{V} = -C'(O)/C(O) = 1/2T1/2$ ml/min/ml, where T 1/2 is the half time for the initial steepest part of the wash-out curve. This T 1/2 is found by plotting the curve on a semilogarithmic paper.

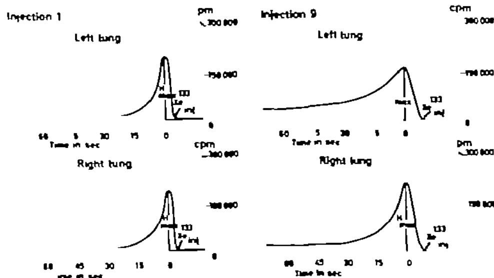


Fig. 2. The same experiment as shown in Table I. To the left simultaneous tracing of ^{133}Xe clearance curves from left and right lung just before rebreathing was started. T the right clearance curves for the tw lungs after period of rebreathing for the left lung. The perfusion was directly related t the H_{max}. As to injection numbers, see Table I

Results

In order to test the method, unilateral hypoxia was produced by rebreathing technique. After a first injection of ^{133}Xe w th measurements as described, a thin foley catheter was inserted through a tracheostoma and under fluoroscopy placed w th its tip in one main bronchus. The catheter was made to fit the bronchus tightly by the ballon around its tip This balloon was filled with a small amount of Isopaque® Nyco which made it possible to control the position of the catheter fluoroscopically The animal was breathing air through the catheter for the first 5 min after its positioning in the bronchus. A new injection of ^{133}Xe was then made The bronchial catheter was then connected to a small rubber balloon containing 5 ml of nitrogen, and rebreathing from this balloon started. ^{133}Xe injections were made at short intervals for the first 30 min (Table I) and thereafter every hr for 3 hrs. Then entilation with a was restored, first through the catheter nd then with removal of the catheter Injections of ^{133}Xe were made every 5 min during these periods. The O concentration in the rebreathing balloon measured after one experiment of this type (Table I) was 7 8 per ent.

Table I and Fig 2 show the result from an experiment of this type The insertion of the catheter in one main bronchus caused a reduction of the entilation in the corresponding lung because of the narrowing effect of the catheter After 5 min f rebreathing the relati perfusion in the rebreathing lung w reduced by .5 per cent onl Further moderate reduction of the relati perfusion of this lung down to 43.5 pr cent of the t tal perfusion occurred in the interval from 1 hr to 3 hrs after

TABLE I Results of ¹³³Xenon radiospirometry i experiment with rebreathing for the left lung

		Time minutes	Per cent perfusion left l ng	Per cent ventilation lef lung	V/A left l ng ml/min/ml	V/A right lung
N catheter	I ject. 1	0	.0.0	50.0	13 3	13 3
With catheter	Inject. 2	5	50 0	22 (	3 6	19 3
R breathing	I ject. 3	10	47 6			20 4
R breathing	I ject. 4	15	48.1			13 3
Rebreathing	I ject. 5	20	48.2			13 3
Rebreathing	Inject. 6	30	48.2			19 0
R breathing	Inject 7	60	48.3			15 0
Rebreathing	Inject 8	120	46.1			13 0
Rebreathing	I ject. 9	180	43 5			18 5
With catheter						
Breathing in ai	I ject. 10	5	46.0	27.3	5.2	13 9
Breathing i air	I ject. 11	10	47.2	37.6	9 5	13 8
N ca heter	I ject. 1	5	48.4	43 8	14 8	19 0
No catheter	I ject 13	10	51 0	54 0	14 8	12 6
No catheter	I ject 14	15	50.9	50 0	18.5	18..

The perfus n of th l ng is expressed i per cent of the total lung perfusion
V is the en lation/ nit lveolar vol me (V)

start of reb eathing When normal ventilation was restored there was a gradual normalization of the distribution of the pulmonary blood flow in the course of 20 min

) Three similar experiments with 3 different rabbits gav nearly identical results The reduction in relativ perfusion of one lung reached during 3 hrs of rebreathing w 8 4 .2 and 6 5 per cent respectively

Duri g the tages ith rebreathing for one lung the ventilation on that side could not be calculated from the wash-out curve since rebreathing of the ¹³³Xe el minated from that lung wou ld occur

Discussion

With the equ pmen t described the method of ¹³³Xe-radiospirometry can be used in rabbits, nd the result ob tained have proved reproducib e Bronchospirometry in small animal i d nsid rable narrowing of the bronchus lumen with influence on the ntilat n nd it will thus disturb steady state evaluations I the present experiment such c th eterization with narrowing of a bronc us was carried out because one ted t test the effect of rebreat ing The ¹³³X technique can, however be applied w th t a y bronc us cathet rization, and it needs thus not influence entilation i ll

The many investigat in the influence f the respiratory gases on pulmonary

circulation, stimulated by the important work of Euler and Liljestrand (1946) have been reviewed by Fischman (1961) There seems to be a prompt vasoconstrictor response to alveolar hypoxia in most species, whereas in the rabbits there is a much less marked and more gradual effect. In rebreathing experiments hyperventilation with alcalosis may to some extent counteract the hypoxic effect on the pulmonary vessels (Lloyd 1966)

The moderate and graduate fall in pulmonary blood flow found during unilateral hypoxia in the present investigation is in agreement with the findings of Durken and Heemstra (1948) This agreement in results which have been obtained with different techniques indicates that the radiospirometric method described may be useful in studies of this type.

For valuable help I am indepted to professor Sven-Eric Lindell and his staff at the Department of Clinical Physiology Malmö Allmänna Sjukhus, Malmö, Sweden.
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Effects of Some Monovalent Anions on Fluxes of Na and K, and on Glucose Metabolism of Ouabain Treated Human Red Cells

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Abstract

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The present work deals with the effects of the anions Cl, Br NO$_3$, I, SCN and HCO$_3$ on Na and K fluxes, and on glucose metabolism of human red cells suspended in isotonic electrolyte media at 38 C and pH 7 40 in the presence of ouabain. When 120 mM Cl of the isotonic medium was substituted by the anion concerned Na influx and K efflux were unaffected by Br increased about 30 per cent by NO$_3$ and I but increased 200 to 250 per cent in the presence of SCN Substitution of 120 mM Cl by HCO$_3$ increased Na influx into red cells by 250 per cent In contradistinction K efflux was reduced by 25 per cent The increase of Na influx was linearly related to HCO$_3$ concentration at pH 7 40

At intracellular Na concentrations between 10 and 35 meq/kg cell water the rate constant of Na efflux was close to 0 15 h in the presence of all 6 anions studied. At external K concentrations from 5 to 7 6 meq/l potassium influx into ouabain treated cells suspended in chloride medium was three as big as the influx calculated from the flux ratio equation $M_i/M_o = K_o/K_i \exp(-EF/RT)$ A similar condition was found in the presence of Br I all other media studied (NO$_3$, I SCN and HCO$_3$) the ratio between the measured fluxes of potassium agreed with the calculated values of flux ratio for passive independent fluxes

A net flux of water into the cells was observed in cells suspended in SCN and HCO$_3$ media In the same media the gain of Na in cells exceeded K loss. The relation of water net flux to the net gain of cations was 6 5- 7 2 g water per meq The rate of glycolysis was unaffected by the substitution of Cl by HCO$_3$ was reduced by 15 per cent in the presence of Br NO$_3$ and I and decreased by 30 per cent in the presence of SCN

Late in the nineteenth century Hofmeister (1888) studied the effects of anions on physical properties of protein solutions. He found that various anions exerted specific effects, and he arranged a sequence of ions indicating the relative molar concentrations required for salting out egg albumin the socalled lyotropic series of anions.

The anions of the lyotropic series have later been shown to affect properties of almost all cell membranes The phenomena displayed by the membranes of excitable

cells have recently been reviewed by Horowicz (1964) Red cell permeability to cations in the presence of various anions of the lyotropic series has previously been investigated by Davson (1940) His results, obtained on cat erythrocytes, prompted him to reject the colloid chemical viewpoint of Höber (1909 1945) that the permeability of the cell membrane is affected by its degree of hydration, which may be determined by ambient anions of the medium.

As will be shown the effects found by us on human red cells differ fundamentally from those found by Davson on red cells of the cat. It is possible that the differing effects of anions on red cell membranes from cat and man reflect differences of membrane structure which underlie permeability to Na and K. The cation composition of red cells from the two species differ principally cat erythrocytes possessing a high sodium and a low potassium content—opposite to the relation found in human red cells.

In this article the effect of monovalent anions of the lyotropic series (Cl, Br NO_3, I and SCN) on Na and K fluxes in ouabain treated human red cells is reported. All results were obtained on red cells incubated at 38° C and at a pH of 7 40 in the presence of various monovalent anions and ouabain. It was found that permeability to both Na and K was increased in the presence of NO_3, I and SCN In addition a study of the effect of bicarbonate was performed because of the biological importance of this ion. The effect of bicarbonate on red cell permeability differed fundamentally from the effect exerted by other anions, bicarbonate increasing permeability to Na but decreasing permeability to K. A preliminary report of some of the results was published previously (Wieth and Funder 1965)

Methods

The technique of incubating red cell suspensions controlled temperature and pH, nd information bout the simultaneous determination of unidirectional fluxes of sodium and potassium by means of the isotopes ^{24}Na and ^{42}K has been reported previously (Funder nd Wieth 1967 b) The same reference contains deta ls bout the chemical nalyses employed, calculations of unidirectional fluxes, and the adv ntages of relating Na, K, and water movements to red cell solids. According to previous findings (Funder and Wieth 1967) trapping of 2.2 per cent extracellular N and K between the isolated cells have been considered in the calculations.

The electrolyte media employed were of the following composition Na 142 mM, K 3 7 mM, Ca 1 5 mM, Mg 1 mM, HCO_3 22 mM Cl 6.5 mM (derived from he addition of $CaCl_2$, $MgCl_2$ nd KCl) phospha 1 1 mM (as K_2HPO_4) and X 120 mM, X representing one of the following monovalent anions Cl, Br NO_3 I SCN, or HCO_3. The bicarbonate was made by titrating an appropriat mount of 1 000 M NaOH with CO_2 The media contained 5—10 mM glucose nd huma albumin 5 g l Reference the anions media is made as g chloride medium" meaning the medium in which 120 mM X is made up by chloride

pCO_2 of he salt solutions was adjusted the level yielding pH of 7 40 38 C 237 mm Hg he bic bonat medium, 36 mm Hg he other media Precipita ion of Ca and Mg carbona in he bicarbonate medium was avoided when addition of $CaCl_2$ and $MgCl_2$ was postponed un il titration ith CO_2 had been completed I as controlled by analysis tha Ca nd Mg ta ed dissolved throughout experiments in bicarbonate medium All salts were of anal ical grade

Procedure Red cells from fiv healthy persons four men and one woma hin the age 25—35 ears were employed hroughout the experimental series Blood samples employed for experiment ere controlled for haemoglobin content, haematocrit alue and reticulocyt and ery hrocyte

counts. All values agreed with normal values previously reported (Funder and Wieth 1966).

50 ml of heparinized blood was centrifuged at 1500 g for 10 min, plasma and white cells were removed, and the red cells were resuspended at a haematocrit of 0.1—0.2 in the appropriate electrolyte medium. This procedure was repeated twice to remove plasma constituents completely. After final centrifugation the cells were suspended in a volume of the salt solution corrected sufficient to make a haematocrit of 0.25—0.40, the relative volume of medium being 0.60—0.75. The washings were carried out at 5—10 °C. By analysis of the anions Cl, Br, SCN and HCO_3, it was ascertained that the washing procedure substituted more than 99 per cent of the original diffusible anions of the blood with the anions of the electrolyte medium. Control counts showed an average removal of 94 per cent (S.E. 1.5, n = 10) of the white cells. After washing, the red cell suspension was incubated at 38 °C and at a pH of 7.40 by the technique previously referred to. Ouabain (0.014 M dissolved in the electrolyte medium employed at the experiment) was added before start of the experiment to make a concentration of 5×10^{-5} moles ouabain per litre cell suspension. When pH and temperature equilibration had been attained 20—40 min after incubating the cells, radioactive tracers were added, initial sample drawn, immediately cooled to 0 °C, and cells and medium isolated. Sampling was repeated with intervals of 60—90 min during the following 5 hrs incubation. Cooling the cell suspension to 0 °C during isolation of the cells was an efficient means of retarding Na exchange in the presence of chloride and bicarbonate. It has previously been shown that Na uptake is reduced by 90 per cent by cooling cells suspended in plasma and chloride medium from 38 to 0 °C (Funder and Wieth 1967 [illegible]). The uptake of labelled Na was determined with the same technique in four of the experiments carried out in the bicarbonate medium. Inhibition of sodium influx proved to be even more efficient in the cold in this medium, both absolutely and relatively, as the uptake was 0.37 meq per kg solids per h —only about [illegible] per cent of the uptake at 38 °C. However in the thiocyanate medium the uptake of labelled Na was only slightly depressed at 0 °C compared to the uptake at 38 °C, the average gain in four experiments being 12.0 (twelve) meq per kg solids per hr. In the 5 experiments reported in Table III the average uptake of labelled Na during isolation of RBC was 3.3 meq Na per kg red cell solids (S.E. 0.16, n = 5). This uptake is commensurate with the time lapse of 10—15 min from sampling until a high degree of cell packing has been achieved during centrifugation (Funder and Wieth 1967 [illegible]). This uptake of Na into the cells during isolation was accounted for in the calculation of Na fluxes.

In all experiments *net fluxes* of Na were determined by direct flame photometric analyses of cell Na. Net fluxes of K were determined by analysis of cells as well as calculated from the increase of K concentration of the external medium (with due consideration to water shifts occurring between cells and medium). Determinations of K net fluxes could thus be controlled by comparing the K loss from cells with the K gain determined by analysis of the medium. For the 15 expts. carried out in the Cl, SCN and HCO media the K content of the cells at the end of experiments calculated from the amount of potassium recovered in the medium averaged 99.4 per cent (S.E. 0.6, n = 15) of the value found by direct analysis of the cells. The relative increase of K concentration in the medium was 50—

TABLE I. Results of repeated determinations of fluxes of Na and K on red cells from two donors. The cells were suspended in the chloride medium described in the method section in the presence of ouabain (5 × 10⁻⁵ M) 38 °C, pH 7.40.
M_i = influx, M_o = efflux.
k_i and k_o are the rate constants of influx and efflux (Funder and Wieth 1967 b).

Donor Expt.	Sodium: net flux (meq/kg solids/5 hrs)	Sodium: k_i (/h)	Sodium: k_o (/h)	Sodium: $M_i = k_i \cdot 142$ (meq/kg solids/hrs)	Potassium: net flux (meq/kg solids/5hrs)	Potassium: k_i (/h)	Potassium: k_o (/h)	Potassium: M_i (meq/kg solids/hrs)	Potassium: M_o (meq/kg solids/hrs)
NJ 22.VIII	16.0	0.0[illegible]3	0.10	9.0	11.8	0.17	0.022	1.16	5.1
NJ 31.VIII	13.6	0.062	[illegible]13	8.8	12.1	0.21	0.023	1.22	5.5
JF 3.VIII.	11.9	0.058	[illegible]1	8.2	12.8	0.21	0.020	1.40	5.5
JF 1.IX.	13.0	0.059	[illegible]13	8.4	11.9	0.21	0.020	1.31	5.3

100 per cent of initial concentration in these experiments. I distinction to this the losses of K from the cells were only from four per cent (HCO medium) to 11 per cent (SCN medium) Determinations of net fluxes in individual experiments were therefore calculated with the highest precision from he increase of extracellular potassium concentration. No corrections were pplied for potassium liberation by hemolysis, since control of the haemoglobin concentration of the medium showed that potassium of this source in all experiments was less than one per cent of net flux.

T estimate the *reproducibility* of determinations of fluxes repeated determinations of K and Na fluxes were performed on cells from tw donors. The medium employed was the chloride containing medium specified bove Determinations of net fluxes and of the rate constant of influx (k_i) and efflux (k_o) are shown in T ble I The reproducibility in experiments of three hours duration was better than the one seen when fluxes were determined in periods lasting one hour (cf standard errors stated in T ble V and VIII)

Flux ratio analysis. An ttempt was made to test the K fluxes by means of flux ratio analysis (Ussing 1960) Pass independent unidirectional fluxes should be rela ed to the aqueous activities of the cations on either side of the membrane by the following equation

$M_i/M_o = A_o/A_i \; \exp\,(-zEF/RT)$

M and M_o indicate influx and efflux respectively A activity of the ion considered, I and denoting inside and outside phase the alency of the ion, E is the electrical potential difference between medium and cells (in Volts) with potential of zero of the medium as reference, F is Faradays constant 96493 C eq R is the gas constant (8.314 Joule K mole^{-1}) and T is the absolut emperature (°K)

Strictly speaking flux ratio analysis can only be pplied to system which is in steady state. I the present experiments cell potassium concentration and potassium efflux may be considered to be relatively constant, whereas K influx increases with external K concentration. However it was found that increase of external K concentration was linearly related time. If k_i does not change during experiment, the alue of K influx at 90 min will therefore represent influx exactly at this time as well as the average influx during the whole experimental period. The results indicated tha k_i was only dependent on external K concentration in the experiments carried out in chloride and bromide media. The change of k_i calculated by means of experimentally determined alues from separat series of experiments was from 0.173 to 0.143 with K_o increasing linearly from 4.9—7.8. Linear interpolation between the two abov alues of k_i gives value of 0.158 be compared with theoretically correct alue of 0.155. I was therefore considered t be justified to perform comparison of measured fluxes with theoretical alues, calculating the fluxes found in the middle of he three hour lasting experiments.

Results

I Sodium fluxes

a) *The effect f Cl, SCN and HCO on N fluxes* The aim of the experimental design was to make the anionic composition of the media the only variable, keeping concentrations of other ions, pH, and temperature constant. Red cell permeability to sod um was appreciably increased n the presence of thiocyanate and bicarbonate when compared to the permeability found in the presence of chloride. Tabl II shows the Na net fluxes. The cells were incubated at 38 C and pH 7 40 in the three media. The average Na concentrations of the media during the experiments were compa able (136—141 meq l lowest in the thiocyanate medium. The gain of N in th presence of thiocyanate nd bicarbonate was close to 40 meq per kg cell solids, t be compared w th an increase of 12 meq Na of the cells suspended n chloride medium Table III hows the initial nd final Na concentrations of th cells together w th the values of labelled Na found n the cell at the end of the experiments Th tabl also shows the rate constants of Na influ k nd f efflux k_o together w th the influx alues calcul ted at an external Na concentration of 142 mM Compared to the value found in chlorid medium 8.2 meq kg cell solids h sod um nflux

TABLE II The effect of Cl, SCN and HCO_3 on net fluxes of Na in human red cells in the presence of ouabain 3 10^{-4}M. (38° C, pH 7 40) The results were obtained after three hours incubation in the various media. Each result is the mean of a series of five donors, S.E. is stated in parenthesis
The electrolyte media are described in the method section

Medium	Na medium (meq/l)	Net flux (meq Na/kg solids/3 hrs)
Chloride	141	11 9
	(0.7)	(0 5)
Thiocyanate	136	37.2
	(2.9)	(1 9)
Bicarbonate	141	39.5
	(0.5)	(1.5)

TABLE III Effect of Cl, SCN and HCO_3 on intracellular Na, radioactively labelled Na, and unidirectional fluxes of Na in human erythrocytes in the presence of ouabain 3 10^{-4}M (38 C, pH 7 40)
Each result is the mean of a series of five donors, S.E. is stated in parenthesis. The results are from the same series of experiments as reported in Table II
M_i = influx. k_1 and k_2 are the rate constants of influx and efflux

Medium	Cell Na (meq/kg solids) 0 hrs	Cell Na (meq/kg solids) 3 hrs	Labelled Na (meq/kg solids) 3 hrs	k_1 (h^{-1})	M (k_1 142) (meq/kg solids/hrs)	k_2 (h^{-1})
Chloride	22 1	34 0	19.6	0.058	8 24	0.14
	0 6)	(1 0)	(0.5)	(0.002)		(0 01)
Thiocyanate	31 4	68 6	50.6	0.147	20 87	0 15
	(1 3)	(2 8)	(1 3)	(0.006)		(0 02)
Bicarbonate	28 6	68 1	49.6	0.144	20 45	0 14
	(2 4	(1 6)	(0 6)	(0 004)		(0 01)

The correction for uptake of Na during the isolation of the cells at 0° C in thiocyanate medium was 3 5 meq per kg cell solids (S E 0 16) cf method section.

was increased by a factor of 2 5 in the two other media. In spite of the presence of ouabain an appreciable efflux of Na persisted in all three media. The rate constant (k_2) of this Na efflux was 0 14—0 15 independent of the medium employed for incubation. k_2 was constant within the range of Na concentration from 20—70 meq kg cell solids (corresponding to 10—35 meq/kg cell water) So whereas Na influx remains constant during the experimental period Na efflux increases steadily with increasing intracellular Na concentration

TABLE IV Net fluxes, and rate constants of unidirectional Na fluxes in human red cells suspended in bicarbonate medium with and without calcium. Results from two experiments on cells from the same donor (Ouabain 3 10^{-4}M, 38° C, pH 7 40)
k_i and k_e are the rat constants of influx and efflux

Donor	Medium	Net flux (meq Na/kg cell solids/3 hrs)	k_i (h^{-1})	k_e (h^{-1})
JW	Bicarbonate (Ca 1 5 mM)	39.6	0.135	0.15
JW	Bicarbonate (Ca 0 mM)	38.8	0.143	0 16

Determination of N fluxes was repeated in a calcium-free b carbonate medium. Table IV shows the values of k_i k and net fluxes found in two experiments on cells from the same donor Total removal of Ca from the medium did not affect Na fluxes.

TABLE V The effect of Cl, Br NO_3, and I on fluxes of N in human red cells in the presence of ouabain 3 10^{-4}M. (38 C, pH 7 40)
Th results were obtained on cells from wo donors in experiments of three hours duration. The experiments were subdivided int one hour periods. The rate constants of influx and efflux (k_i and k_e) are the means of three determinations, S.E. is stated in parenthesis
The electrolyte media employed re described in the method section

Donor	Medium	Cell N (meq/kg solids) 0 hrs	3 hrs	Net flux (meq/kg solids/3 hrs)	Labelled N (meq/kg solids) 3 hrs	k_i (h^{-1})	M_i (k_i 142) (meq/kg solids/hrs)	k_e (h^{-1})
JF	chloride	20.7	32.7	12.0	19 5	0.058 (0 002)	8 2	0.15 (0.01)
JW	chloride	21 7	33.6	11 9	17.8	0 050 (0.002)	7 1	0.11 (0 03)
JF	bromide	20.3	32 8	12.5	18.9	0 053 (0.002)	7.5	0 13 (0.03)
JW	bromid	25 0	37 0	12 0	18.1	0.048 (0.003)	6.8	0.10 (0.03)
JF	nitrate	21 4	39 8	18 4	25 7	0.074 (0 004)	10.5	0.14 (0 01)
JW	nitrate	24 9	40 1	15 3	23 6	0 068 (0.003)	9 7	0 14 (0.03
JF	iodide	26 9	43 8	16 9	25 9	0.074 (0 007	10 5	0 14 (0 06
JW	iodide	22 5	40 4	17 9	22 4	0 064 0 001	9 1	0 08 (0.04

b) *The ffect f B NO_3, and I on N fluxes* Since chlorid nd thiocyanat represent the outer ranges of the lyotropic series of monovalent nions, t was in estigated if the ions of intermediate location in the series i.e. Br NO_3, and I exert any effect

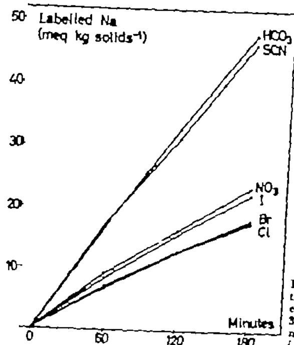


Fig 1 Effect of al monovalent anions on the uptake of labelled sodium by human erythrocytes. (pH 7 40, 38 C, ouabain 3 10 M Donor J W) The electrolyte media indicated on the figure are described i the method section.

on the transfer of Na. Table V summarizes the results obtained on cells from two donors. Data obtained in chloride medium on cells from the same donors are presented for the sake of comparison It appears that sodium influx in chloride and bromide containing media was of the same magnitude. However in the nitrate and in the iodide media Na nflux was increased by approximately 33 per cent. The net flux of sodium increased correspondingly in these media Tabl V renders no evidence that the rate constant of Na efflux (k) is changed in the presence of Br I or NO_3.

Fig 1 shows the amounts of labelled Na found after 1 2 and 3 hrs incubation in the presence of the six anions considered. Th results of the figure were obtained on cells from the sam donor The figure gives a clear impression of the differing effects of a ious anions on Na influx ndicating the sequence Cl = Br < NO = I < SCN = HC O

c) The elation betu een Na influx and SCN or HCO_3 concentration of the medium. Fig 2 shows the alue f Na i flu found n an experiment in wh ch SCN concentration was varied from 0 90 mM keeping the sum of Cl and SCN constant (120 mM Na influx aried l n rly with the SCN concentration of the medi m the influx found by extrapolat ng to SCN = 120 mM was 16 3 meq kg solids h⁻¹ somewhat lower than the average alu shown in Table III

Bicarbonat concentration was ried between 2 and 142 mM in a series of experiments on cells from one donor Fig 3 The increase of Na infl x displayed a linear correlation to bicarbonate concentrat on

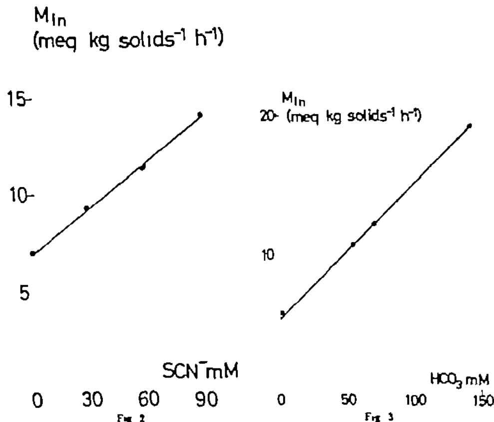


Fig 2

Fig 3

Fig 2. Sodium influx as function of thiocyanate concentration of the medium. (pH 7 40, 38° C, ouabain 3 10 M Donor J W) The shown concentrations of SCN were obtained by mixing appropriate volumes of the chloride and thiocyanate media described in the method section. The bicarbonate concentration was 22 mM all thiocyanate concentration. The regression line indicates the relation M_{in} = 0 078 (SCN) + 6 98

Fig 3. Sodium influx as function of bicarbonate concentration of the medium (pH 7 40 38 C, ouabain 3 10 M, Donor J W) Concentrations of HCO above 22 mM were obtained by mixing appropriate volumes of the chloride and bicarbonate media described in the method section The medium containing only 2 mM HCO was prepared by substituting 20 mM HCO of the chloride medium by chloride. The regression line indicates the relation M_{in} = 0,098 (HCO) + 5.24

The changes in sodium permeability found in the presence of thiocyanate and bicarbonate were completely reversible In one experiment red cells were washed thrice in the SCN medium. After 30 min incubation the thiocyanate was removed by washing the cells 5 times with the chloride medium Sodium influx at 38 C and pH 7 40 was thereafter determined during 3 hrs incubation The complete removal of thiocyanate was substantiated by chemical analysis The rate constant of sodium influx k was 0 047 h^{-1} representing an influx of 6 7 meq kg solids h The corresponding values obtained on cells from the same donor in the chloride medium without pretreatment with thiocyanate were k 0 050 h sodium influx 7 0 meq kg solids^{-1} h

TABLE VI Effect of Cl, SCN and HCO on net fluxes and unidirectional fluxes of potassium in Each result is th mean of series of five donors, S.E. is stated in parenthesis media re described in the method section
M_I nd M_O influx nd efflux
For explanation of the calculation of the flux ratios see method section

Medium	Cell K (meq/kg solids)		Net flux meq/kg solids/3 hrs)	Ke (meq/l)		
	0 hrs	3 hrs		0 hrs	1.5 hrs	3 hrs
Chloride	264 4	252.6	—11.8	4.9	6.2	7.6
	(4 0)	(3.9)	(0.5)	(0 3)	(0.4)	(0 4)
Thiocyanat	249.3	221.5	—27.8	5.2	7 7	10 2
	(4 1)	(3.5)	(0.9)	(0.1)	(0 1)	(0.2)
Bicarbonate	252.2	241.5	—10.7	5 6	6.8	8.1
	(5.5)	(5.7)	(0 4)	(0.6)	(0.6)	(0 6)

TABLE VII The effect of Cl, Br NO_3, and I on net fluxes and unidirectional fluxes of K in the The results are derived from the same series of experiments as reported in T ble V one hour' duration, S.E. stated in parenthesis. K_e potassium concentration of the

Donor	Medium	Cell K (meq/kg solids)		Net flux (meq/kg solids/3 hrs)	K_e (meq/l)
		0 hrs	3 hrs		1.5 hrs
JF	chloride	276.2	263 4	—12.8	5 65
JW	chlorid	269 9	258 0	—11 9	7.0
JF	bromide	248.9	237 7	—11 2	5.75
JW	bromid	241.2	230.9	—10.3	6.65
JF	nitrate	240 0	220.8	—19.2	7 45
JW	nitrate	256 5	232.5	—24.0	7 75
JF	iodide	249 2	227.3	— 1 9	7 15
JW	iodide	248 0	231 4	—16.6	7.0

human erythrocytes in the presence of ouabain 3 10⁻⁴M, (38 C, pH 7 40)
The results are derived from the same experimental series as reported in Table III. The electrolyt
K_e potassium concentration of the medium
k_i nd k_e rate constants of influx and efflux

k_i (h⁻)	k_e (h⁻¹)	Average fluxes		Flux ratios	
		M	M_e	M / M_e	M / M_e
		(meq/kg solids/hrs)		measured	calcula ed
0.186	0.020	1 14	5.07	0 225	0.069
(0.013)	(0.001)	(0.07)	(0.15)	(0.010)	
0.127	0.043	0.98	10.23	0.096	0.088
(0.003)	(0 001)	(0 02)	(0.31)	(0 004)	
0 047	0.016	0.32	3.88	0 082	0.083
(0 002)	(0 001)	(0.03)	(0.15)	(0.006)	

presence of ouabain 3 10⁻⁴M (38 C, pH 7 40)
k_i, the rate constant of influx, is reported as the mean of three determinations in periods of each medium

k_i h)	k_e (h	Average fluxes		Flux ratios	
		M_i	M_e	M / M_e	M_i / M_e
		(meq/kg solids hrs)		measured	calculated
0 213	0 070	1.20	5 47	0 215	0.062
0 005					
0 137	0.019	0.96	4 93	0 193	0 072
0 009					
0 193	0 070	1 12	4 85	0 231	0.063
0 018					
0 14	0 019	0 94	4 37	0 215	0 073
0 019					
0 07	0 030	0 36	6 96	0 060	0.082
0 01					
0 057	0 033	0 44	8 44	0 030	0 050
0 007					
0 068	0 033	0 49	79	0 0 1	0 8
0 004					
0 063	0 025	0 44	5 97	0 0 4	0 0 2
0 003					

2 Potassium fluxes

a) *Fluxes of K in the presence of Cl SCN and HCO_3.* The fluxes of potassium in ouabain treated red cells were also affected by the anions of the medium. Table VI shows the K fluxes in Cl, SCN and HCO media from the series of experiments of which the Na fluxes were reported in Tables II and III An effect of the anions is apparent merely by inspection of the net fluxes after three hours incubation in the various media The erythrocytes suspended in SCN medium lose almost 30 meq K per kg solids, whereas the cells of the other two groups only lose 11 to 12 meq K during the same period. Investigation of the unidirectional fluxes of K revealed more differences between the effects of anions on K exchanges. It has previously been established that about one fifth of K influx persists in red cells in which digitalis-glycoside sensitive potassium transport is inhibited with ouabain (Funder and Wieth 1967 b) An exchange diffusion of the type originally suggested by Ussing (1947) to be responsible for part of the Na transfer through the muscle membrane, has been suggested to be responsible for the calculated deviations from the flux ratio for passive independent fluxes (Glynn 1956) In Table VI the flux ratios found in the experimental series have been compared to calculated theoretical values as described in the method section. The values inserted for potassium concentrations were the average values found after 90 min incubation The value employed for the potential difference between cells and plasma (—11 1 mV) was the value previously derived by us from the distribution of chloride between cells and plasma at a pH of 7 40 and 38 C (Funder and Wieth 1966 b) Comparison of measured and calculated values shows that experimentally determined flux ratio for cells suspended in a chloride medium was about 3 times larger than the calculated theoretical value (Table VI) In contradistinction to this the measured flux ratios in SCN and HCO_3 media agreed well with calculated values. The ouabain insensitive potassium influx disagreeing with the calculated flux ratio is apparently dependent on the presence of chloride (or as shown later bromide)

b) *The effect of Br NO_3, and I on K fluxes* Table VII shows the fluxes of K in cells from two of the donors as found in the presence of bromide, nitrate and iodide As in Table V the results obtained in chloride medium on cells from the same donors are stated for comparison No differences were found between the magnitude of K fluxes in bromide and in chloride media A discrepancy between experimentally determined and calculated values of flux ratio was accordingly also seen in the presence of bromide However in the presence of nitrate and iodide the agreement between the two values of the flux ratios is as good as in the bicarbonate and thiocyanate media. Potassium efflux was moderately increased both in the presence of iodide and nitrate the loss per hour being 6—8.5 meq per kg cell solids compared to an average potassium efflux of 5 1 meq in the chloride medium (Table VI)

Summarizing the effect of the lyotropic series of anions on the main routes of passive cation transfer Na influx and K efflux, these fluxes were both affected to a similar degree by other anions than chloride. The sequence arranged according

to the effect on the magnitude of these fluxes, was Cl = Br < NO = I < SCN. In contradistinction to this bicarbonate affected Na and K fluxes differently increasing Na influx to a similar degree as SCN but decreasing K efflux to values 25 per cent smaller than those found in the presence of chloride.

3 The relation between net fluxes of cations and water shifts

Cells suspended in the salt solutions in the presence of ouabain invariably lost potassium and gained sodium. Table VIII shows the relation between changes of water contents and net fluxes of Na and K of the cells incubated in Cl, SCN and HCO media. In the chloride medium sodium gain and potassium loss almost exactly balance each other. Consistent with this finding the changes in water contents during three hours incubation of red cells in a chloride medium are not significantly different from zero. Oppositely the cells suspended in the presence of SCN or HCO exhibit a net gain of cations during incubation, sodium gain in both cases exceeding potassium loss. However the net gain of cations as well as the net gain of water of cells in bicarbonate medium are thrice the values seen, when cells are suspended in thiocyanate medium. When the net gain of cations (meq/kg solids) is related to the net gain of water (g/kg solids) water net flux equals 6.5—7.3 g water per meq cation gained.

In spite of the fact that the sodium concentration and the osmolality of the media are identical the initial water content of the cells differ (Table VIII). The higher water content of red cells suspended in a bicarbonate medium as compared to cells suspended in a chloride medium is explained by the fact that the cells have been incubated 20—40 min at 38° C in an equilibration period before start of experiments.

TABLE VIII. The relation of water net flux to net fluxes of Na and K in red cells suspended in chloride thiocyanate, and bicarbonate media in the presence of ouabain 3 · 10^{-4}M. (38° C, pH 7.40)
Each result is the mean of series of five donors. S.E. is stated in parenthesis. The results are derived from the same series of experiments as reported in Table II and VI.

Medium	Initial cell water content (g H_2O/kg solids)	(1) cell water net flux (g H_2O/kg solids/3 hrs)	Net fluxes Na (meq kg solids/3 hrs)	Net fluxes K (meq kg solids/3 hrs)	(2) (Net flux Na) + (Net flux K) (meq kg solids, 3 hrs)	$\frac{(1)}{(2)}$ g H_2O/meq
Chloride	1,849 (33)	5 (9)	11 9	—11 8	0 1	—
Thiocyanate	1 761 (21)	69 (7	37 2	—2 8	9 4	7 3
Bicarbonate	1,932 (37)	187 (7)	39.5	—10	8 8	6.5

TABLE IX. Glucose utilization and lactate production of h ma red cells suspended in chloride, thiocyanat and bicarbonat media in th presence of ouabal 5 10 M. (38° C, pH 7 40)

The results are derived from the same series of experiments as eported in T ble III and VI the esults from four additional experiments performed in hloride medium were included. S.E. and number of experiments re stated in parenthesis

Med um	Glucose consumption (mmole/kg cell solids/hrs)	Lactat production (mmole/kg cell solids/hrs)	mmole Lactate / mmole Glucose
Chlorid	3.29	6.26	1.88
	(0.11 −9)	(0 24 −9)	(0 03)
Thiocyanat	2.20	4 13	1 91
	(0.13 −5)	(0 15 −5)	(0.14)
Bicarbonat	3.30	6.34	1 92
	(0.19 −3)	(0.44 −5)	(0.06)

For the same reason it is astonishing that cells suspended in the SCN medium have a lower initial water content than the cells incubated in chloride medium A similar decrease of water content was found in cells suspended in bromide iodide and nitrate media The a erage initial water content of the 6 expts reported in Table V and VII wa 1733 g per kg cell solids (range 1681—1795 g/kg cell sol ds)

4 Glucose metabolism of red cell suspended in media f varied anionic composition.

Table IX ummarizes data on the glucose metabolism of red cells suspended in Cl, HCO_3, and SCN media Both glucose consumption and lactat production are reduced by one third in the presence of SCN In chloride and bicarbonate media th rates of glucose metabolism are identical glucose consumption being 3.3 mmoles kg c ll solid h The rat o of lactate produced to glucose ut lized indicates that metaboli m is almost ex lusively anaerobic, 95—97 per cent of th glucose being metabol ed to la t te u all media

Lactat p oduction w mea ured in the six experiments carried out in Br NO and I media Th res lt fr m these experiments all fell within the range 4 1 to 5 6 mmoles la t t produced per kg cell solids a d hour As no difference was evident between tl tes of la tat production in these three media the results were subjected to m m t ti tical t eatment A erage lactate production was 5 0 mmoles kg cell s l d l 5 1 0 24 n (This alue i intermediat t the values for cells in chlorid l th anate media Table X and d ffer significantly from both (both p alue b q 0 05 Glucose metabol sm of ouabain treated red cells thus decreases when lorid is subst tuted by other monovalent anions of the lyotropic series at const t ternal pH wherea the replacement of chloride by bicarbonate is without ome qu nces t the rat of glucose turno er

Discussion

Our experiments showed that potassium efflux and sodium influx were increased in the presence of iodide, nitrate and thiocyanate (Table II through VII) The permeability to sodium and potassium was increased to the same degree by these anions sodium influx, potassium efflux, and net fluxes of the two ions all being increased by 250 to 300 per cent, when 120 mM chloride in the medium was substituted by 120 mM thiocyanate.

The effect of the lyotropic series of anions on the exchange of sodium and potassium in red cells has previously been studied on cat erythrocytes by Davson (1940) He determined the net fluxes of sodium and potassium in cells which were suspended in 0 165 M solutions of potassium salts of the various anions. The results differed fundamentally from our findings on human cells, the main difference being that sodium and potassium fluxes were affected oppositely The hydrating anions of the Hofmeister series (iodide and thiocyanate) thus caused an increased permeability to K and virtually stopped Na loss. Ions of the dehydrating end of the series (sulfate and chloride) exerted an opposite effect, increasing sodium loss and decreasing the influx of potassium. These results lead Davson to reject the theory that the effect of the anions on membrane permeability was predictable from a knowledge of their ability to hydrate or to dehydrate colloidal systems as had been maintained by Höber (1909 1945) Davson suggested that the SCN ion modifies membrane permeability in virtue of its adsorbability at the protein water interface.

However it is still a matter of conjecture how the anions affect membrane permeability We suppose that they act on the properties of the cell membrane itself although more evidence is needed to exclude that e.g the activity coefficients of the intracellular ions are changed in the presence of various anions. The literature shows much experimental evidence that the lyotropic series of anions may interact with all components of the complex architecture of the cell membrane. The following examples show that lipids, proteins, and even the solvent water of the system all must be included as probable sites of action for the anions. Electrolytes may affect the thickness of the water layer found between bimolecular leaflets of cephalin (Overbeek 1952) Poorly hydrated anions as iodide and thiocyanate are firmly bound to the quarternary ammonium groups of phospholipids, and the structure of lecithin micelles is changed by anions of the lyotropic series (Booij and Bungenberg de Jong 1956) Anion series similar to that reported in our work have been found in studies of physico-chemical properties of pure protein solutions Hippel and Wong 1964 noted that the structural stability of several protein molecules was decreased by ions in the following sequence Cl < Br < NO < I SCN The helices of collagen, myosin, and of deoxyribonucleic acid are easily disrupted in the presence of iodide or thiocyanate Hippel and Wong 1964 Warren Stowring and Morales 1966 Hippel and Wong concluded that the effect of anions on macromolecular structure is a consequence of very general effects on the structure of the solvent which in turn modify solvent-macromolecular interaction necessary for the stabilization of mole

cular structure. The general applicability of this hypothesis has been questioned by Robinson and Jencks (1965) who pointed out that a lacking effect of the lyotropic series of cations (Li, Na, K, Cs) on the properties of proteins, and the finding of a negative entropy of interaction between anions and a peptide was incompatible with an effect mediated by a salt induced disruption of water structure. In agreement with other investigators they considered the physical state of proteins to be changed by binding of anions to positively charged groups. The binding of anions to proteins follow the sequence of affinity to anion exchange resins $Cl<Br<NO_3<I<SCN$ (Sollner 1949 Peterson 1954 Scatchard and Yap 1964) An affirmation of the effect of anion adsorption on cation permeability of the red cell would support the conception of the erythrocyte membrane as a positively charged perm-selective membrane (Tosteson 1955)

It has been suggested (Davson 1940) that the influence of anions on fluxes of Na and K at least in part may be due to diffusion potentials caused by differences of membrane permeability to various anions. From several works it is known that the anions considered in this study equilibrate rapidly between cells and medium (Ege 1922, Maizels 1934 Luckner 1939 and Tosteson 1959) It was confirmed by chemical analysis that the distribution of chloride, bromide, thiocyanate, and bicarbonate had reached equilibrium before the start of the experiments.

It is only possible at present to assign hypothetical points of action to the effect of anions on cation permeability of the red cell membrane. Prospective investigations must be designed to distinguish between effects of ion adsorption, effects on the degree of hydration of the membrane, and possible effects on the structure of the solvent water

The relation between influx and efflux of potassium

In the presence of nitrate iodide thiocyanate, and bicarbonate calculated flux ratios agreed with the ratio of measured unidirectional potassium fluxes. In the presence of chloride and bromide potassium influx was greater than predicted from the independency relation $M_i = M_o\ K_o/K_i\ \exp(-zEF/RT)$ Postponing scepticism to the validity of the calculated flux ratios for a while the simplest interpretation of the above results is that the exchange of potassium in the presence of ouabain is mediated by passive uncoupled ion movements, when the system is dominated by other monovalent anions than chloride and bromide Apparently some additional transport mechanism is activated in the presence of the latter two anions There can be no doubt that all the ouabain sensitive potassium influx was inhibited under our experimental conditions. The ouabain concentration employed (3×10^{-5} M) is about 100 times the concentration necessary to ensure maximal inhibition of K influx (Solomon, Gill and Gold 1957) In addition we have found that potassium influx in the presence of chloride is unaffected by a further increase of ouabain concentration to 2×10^{-4} M Both the findings of Glynn (1957) and of Whittam and Ager 1965 indicate that potassium influx in the presence of ouabain and chloride exhibit saturation kinetics of the Michaelis-Menten type

This might agree with the concept of a carrier mediated transport mechanism, al though it is by no means a proof. Hoffman and Kregenow (1966) have recently suggested that part of the potassium influx found in the presence of ouabain is transported actively However the conception of Glynn (1956) that there is a passive exchange diffusion of potassium across the human red cell membrane is not less likely Both these explanations agree with the flux ratios found by us in the presence of chloride or bromide.

The above conclusions can only prove true if the assumptions made in the calcula tion of flux ratios are legitimate. Some of the possible sources of error must therefore be recalled 1) The justification of calculating membrane potential from chloride distribution may be questioned (Funder and Wieth 1966 b) although t is supported by the fact that the calculated membrane potential of normal red cells agree with the value found by direct measurement (Lassen and Sten Knudsen 1966) 2) The possible effect of a solvent drag on the ion fluxes should also be considered but the difference between calculated and measured flux ratios of cells suspended in the chloride me dium cannot be attributed to a solvent drag effect, because there was no net flux of water in these experiments (Table IX) 3) The last source of error to be dealt with is the substitution of ion activities by ion concentrations in the flux ratio equa tion. This is only permissible if the acti ity coefficients of intra and extracellular potassium ions are equal. In the presence of chloride the ratio of th measured fluxes exceeded the calculated flux ratio by a factor of 3 (Table VI) If identity and incongruity between measured and calculated flux ratio were caused by changes of the intracellular activity coefficient of potassium, t follows that this ctivity coeffi cient should be severely depressed in the presence of chloride or bromide, but almost unaffected by the presence of nitrate iodide, thiocyanate or bicarbonate. Such an effect of chlorid and bromide is highly unlikely There is some supporting evidence that activity coefficients of ions on the two sides of the red cell membrane d not differ much. For chloride this is suggested by the previously mentioned gree ment between values of membrane potential calculated from chloride distribution nd alues obtained by direct measurement. Similar evidence is not available for the activity coefficients of potassium, but in the case of sodium the intracellular concentration determined with a cation selective glass electrode has been found to agree with th concentration found by flame photometry when the intracellular act ty coefficient was assumed to be 0 76 — equal to that of a NaCl solution at an ionic strength of 0.15 The results obtained with the two methods on red cells from 22 normal individuals were 15 1 (S E. 0.4) and 14 8 (S E. 0.3 meq/kg cell water (Clausen et al 1966)

The ffect f bicarbonate on sodium and potassium fluxes

Bicarbonate exerts a specific effect on sodium and potass um fluxes the sense that sodium influx markedly increased wherea potass um efflux is decreased in th pres ence of 142 mM bicarbonate Table III and V This effect w probably noted by Harris and Prankerd (1953) when they found that the rate of sod um exchange a

Ringer solution containing 27.5 mM bicarbonate was faster than the exchange found when human red cells were suspended in a phosphate buffered medium. They ascribed the difference to difficulties of controlling the pH of the medium, but added that "some specific effect of bicarbonate is not excluded".

It is not certain that it is the bicarbonate ion which alters the permeability of the red cell membrane. The substitution of chloride by bicarbonate is inevitably accompanied by an increase of carbon dioxide tension and of carbonate concentration when pH is kept constant. We have previously published results, which render it unlikely that the high carbon dioxide tension is responsible for the increased permeability to sodium, as sodium influx into red cells suspended in a bicarbonate medium was found to decrease with increasing pCO_2 (Wieth and Funder 1963). Among the more likely causes an interaction of carbonate ions with calcium of the membrane must be kept in mind. Mullins (1959) presented evidence that calcium may influence the distribution of pore sizes in cell membranes. He suggested that ions only penetrate pores that they fit closely. During penetration the water molecules of a hydrated ion are replaced by groups of the pore wall. Applying similar considerations to the system examined by us an interaction between carbonate and calcium of the membrane might thus increase the ability of the membrane pores to "solvate" sodium ions and simultaneously decrease the permeability to potassium. The fact that complete removal of calcium from the medium had no effect on sodium influx does not exclude that calcium ions bound in the membrane phase are critical for the permeation of cations. Hoffman (1962) reported that removal of calcium from isolated red cell membranes ("ghosts") is followed by a decreased permeability to potassium, but the effect on sodium influx was not examined.

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Acta physiol. scand. 1967 71 186—193

From the Institut of Physiology and Medical Biophysics, University of Uppsala, Sweden

Interference of Light on the Determination of Low Glucose Concentrations with Glucose Oxidase

By

Bo Abbro

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Abstract

Abbro B *Interference of l ght on the determination of low glucose c ncentrations with gluco e oxidase* Acta physiol. scand. 1967 71 186—193

A method for measuring glucose concentrations down to 10 µM/L is described. This is modification of a method based on commercially available glucose-oxidase reagent. Some factors which re important for good reproducibility were investigated. Most important is the light sensitivity of the chromogenic reaction. The absorbance decrease which results from illumination is, however reversible in the dark. If the samples re kept in darkness ntil the absorbance is measured nd suitable photometer is used the method has good reproducibility and is simple to perform.

Keilin and Hartree (1948 1952) in their extensive investigation, showed that glucose oxidase has a very high pecificity for glucose In 1956 Keston (1956) and Teller (1956) described a method for the determination of glucose with this enzyme which after some modifications became a rapid and reliable technique suit abl for linical use (Levin and Linde 1962, DeVerdier *et al* 1964) The reaction takes place n two stages. Glucose is first oxidized to gluconic acid and the hydrogen so liberated then react with the dissolved oxygen to form hydrogen peroxide the cof tor fl in adenine-dinucleotide (FAD) being involved (Keilin and Hartree 1948 Keil n nd Hartree 1952) In the next stage of the reaction the hydrogen perox de idizes reducing chromogenes to a coloured product whose concentration is determi ed photometrically This reaction is not specific (Hjelm and DeVerdier 1963) and he d st rbed by a n umber of factors. Oxidizing subst nces other than hydrogen per id ma react with the chromogen.

In the e pe e t reported here a commercially a ailable reagent, Glox® is supplied in dr f zen stat and is primarily intended for clinical use

The aim of th work reported here was to dapt the method as described by Kabi (Kabi 1961 f the determination of glucose in concentrations a low a 10 µm/l and als t n our cause of interference with the reaction.

[1] Manufactured by AB Kabi Stockholm 30 Sweden, who generously provided the Glox® used in these experiments.

Materials and methods

The method of Kabi

1.0 ml of the precipitating reagent and 0.05 ml capillary blood are put in series of test tubes. After centrifuging 0.5 ml of the supernatant is removed and mixed with 5 ml Glox® sol tion (see below). The absorbance t 440—460 nm is measured after 1 hr against a blank which is prepared by mixing 0.5 ml of the precipitating reagent with 5 ml Glox® sol tion. A calibration solution prepared by substituting the blood by 0.05 ml of glucose solution (300 mg/ml) is also used.

The precipitating reagent (0.33 M perchloric acid buffered to pH 2.7 with glycine) and the standard glucose solution can be obtained from Kabi. Freeze-dried Glox® is supplied in flasks containing sufficient for 15, 75 or 200 analyses. It is dissolved in water prior to use, about 5 ml of water being used for the amount of substance required for single determination.

Modified method

The precipitation stage was omitted, since the samples did not contain protein. To obtain the optimal pH for the reaction, about 6.5, 1 ml 0.1N HCl was added instead of the protein precipitant to each sample. For determinations in the range 10—100 µM/l glucose, 3 ml of sample was mixed with 1 ml of Glox® at a concentration about five times that normally used (i.e. 80 ml H_2O is added to a flask containing sufficient material for 75 determinations, instead of the usual 380 ml).

For determinations in the range 100—500 µM/l glucose, 1 ml sample was mixed with 3 ml of solution containing sufficient dry Glox® for one determination (i.e. 230 ml added to the flask for 75 determinations).

Two standard samples were included in each series of determinations. The standards contained, in the range 10—100 µM/l glucose 50 and 100 µM/l glucose and in the 100—500 µM/l range 250 and 500 µM/l glucose. These standards were made from a 1 mM/l glucose solution containing 0.002 % (W/V) of the preservative chlorhexidine gluconate (Hibitane® *Imperial Chemical Industries, London, England.*)

Blank samples containing distilled water instead of glucose. All samples were incubated in dark for 45 min 37 C and then stored in the dark (at room temperature) until measurement of the bsorbance at 444 nm.

Tat l

The effects of temperature variations, light, pH and impurities were studied.

Results

The ffect of h at light and pH

Levin nd Linde (1962) found that although the maximal colour intensity developed was independent of temperature the rate of colour development was temperature dependent. While the maximal absorbance was not reached until after 70 min at + 18° C this took onl 40 min at + 37 C. The temperature did not, however affect the maximum alue In the present study t was found that absorbance did not attain stable alue After placement of the sample in the spectrophotometer beam the bsorbance increased. It has also been reported (Hjelm and DeVerdier 1963) that in strong illumination the colour of the sample fades. Colou hanges during exposure to the spectrophotometer beam may be due either to light or heat.

Samples of 100 µM/l glucose were therefor incubated w th gl se oxidase reagent for one hr at + 3 C and the following experiment w re then performed on the incubated samples

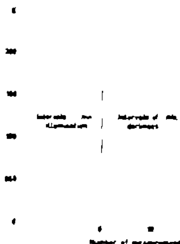


Fig. 1 Effect of illuminating a sample for one minute between absorbance measurements, following by storage in the dark during measurements. The time required for measurements was not constant so that the abscissa is not an exact time scale. See text for f rther details.

(1) A sample was illuminated with a 40W fluorescent tube at a distance of 5 cm, being removed to the spectrophotometer after each min of illumination for measurement of absorbance, which decreased as a result of this procedure. When the absorbance had reached a steady state minimum value the cuvette was kept in complete darkness between absorbance readings, which were made at one min intervals the absorbance then increased again (Fig 1)

(2) A sample as placed in a Beckman B spectrophotometer connected to a Sargent recorder after being exposed to light from an ordinary 60W table lamp at a distance of 10 cm for ? min. The absorbance increased, reaching a stable level after about 10—30 min (Fig 2) The sample was then reill minated as before for ? min and absorbance again sank and then recovered again in the dark to the same level A sim lar experiment showed that the effect of illumination for 1 1 2 min wa apparent for up to 30 min afterwards.



Fig 2 Absorbance recovery after illumination (Beckman DB, Sargent recorder) See text for further details.

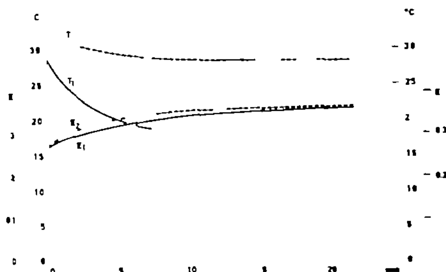


Fig. 3. Effect of temperature on the absorbance recovery after illumination. E and Ea are the absorbancies of the same sample monitored t different temperatures (T_1, T). See text for f rther details.

(3) A thermosond enclosed in a stainless steel injection needle was placed in the sample cuvette at the side of the position of the light beam for continuous recording of the sample temperature The absorbance was measured both with and w thout coo ing of the thermostate bath. The results are shown in Fig 3 where t can be seen that the final absorbance was not temperature dependent. There w s also little difference in the rates which the maximum absorbance was enched, though this did possibly occur more rapidly t the higher temper ature

(4 To test the influence of the illuminating spectrophotometer beam at 444 nm, some samples were prepared in absolut darkness. They were then read repeatedly for 20 min at 444 nm no hange in the absorbance was noted.

(5 F rther samples were read first fter preparation in the dark. They were then illuminated by a 40W fluorescent tube at a d'stance of 5 cm for 1 min The absorbance was reduced and the initial alue was regained after 20 min in the cuvett holder despit cont nual illumination at 444 nm.

(6 The effect of pH on the light induced absorbance decrease w tested by adding either HCl or N OH t cuvettes containing samples. The mount w 1 ml nd the concentrations were for HCl 0 5 N 0 25 N 0 1 N 0 05 N 0 025 N and NaOH 0.05 N 0 1 N 0 25 N 0 5 N nd 1 N W th 0 5 N HCl the pH of the sample wa 68 and illumination by 5W t ngsten lamp distanc f 15 cm had no detectable influence n the absorbance but the sample w bleached to some extent At higher pH alues the bsorbanc recovery afte llumination was lower the higher pH a showed Fig 4



Fig 4 Influence of pH on the absorbance recovery after illumination for 2 min. See text for f rther details

Choic of ph tometer

Since light has a bleaching effect on the end-product of the glucose-oxidase reaction, t seems likely that photometers where the filter is placed between the source and the cuvett will be more reliable than instruments where the filter is between the cuvette nd the detector and in which the cu ette receives unfiltered illumination from the source In order to check this interference, samples were read in a Beckman DU spectrophotometer before and after 5 min illumination by light of different wa elengths in the cuvetteholder with a fully opened slit As seen in Table I in general light of wa elengths between 500 and 600 nm had effect on the sample

T I Effec of illumination of the sample for 5 min I Beckma DU spectrophotometer th ungsten lamp Sl width 2.0

W -length m	Bleeching effect	W e-length m	Bleeching effect
400	—	520	+ +
410	—	540	+ + +
420		560	+ +
430	—	580	+ +
440	)	600	+ +
4.0		625	—
460		650	—
470		675	—
480		700	—
490		750	—
500		800	—

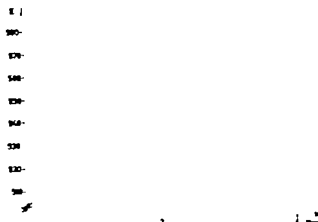


Fig 5 Absorbance decrease in Linson 3 photometer

Since the cut-off at shorter wa elengths may be due to the low intensity of the tungsten source used in these wa elengths, rather than to a decreased sensitiv ty of the sample tests were made w th a higher intensity source A sample was illuminated by light from a Hg-lamp, filtered through a combination of coloured glass-filters which allowed through only light between 400 and 530 nm. After 5 min the absorbance was reduced by 10 per cent.

A series of samples were also illuminated in a Beckman DL spectrophotometer by a H_2-lamp The Wa elengths 365 410 434 486, 656 and 540 nm were tested No reactions was observed except at 540 nm where a small absorbance decrease occurred.

For comparison one sample was lso read in a Linson 3 photometer with 450 nm filter The absorbanc was reduced as shown in Fig 5

Since light, at lea t between 400 and 600 nm may disturb the sample it s d visable to use only photometers with a relatively low light energy and with the light filtered before passing through the sample.

Impurities

This method was intended for use w th experiments in which raffinose was also present. Tests were therefore made to find out if glucose oxidase reacts with the glucose residue of raffinose Raffinose was therefore incubated with glucose oxidase and it required 24 hrs for the colour development to reach the same intensity as that of glucose solution whose mola concentration was 1 per cent of that of the raffinose It is therefore not clear wether this colour was due to oxidat on of raffinose or a tooxidation of the reagent In any case under the incubation onditions sed for glucose determination, raffinose does not interfere

The second stage of the rea tion ma be affected by gents whi h re commonly present, such as traces of alcohol or bicromatic sulph ric cid remaining after w shing procedures, dust, or arious growth inhibiting agent The ffects of some

such agents were tested by adding to samples containing 250 μM/l of glucose, 0.1 ml 95 alcohol, a drop of bichromate sulphuric acid (pH 3) about 5 mg of dust and varying concentrations of chlorhexidine gluconate to the water with which the glucose was diluted No significant effect of small quantities of alcohol in the sample was observed. Small amounts of bichromate sulphuric acid disturb the reaction, and therefore careful rinsing of all glass apparatures after washing in this acid is recommended. It has also been reported (Kabi 1964) that several synthetic detergents interfere and careful rinsing of all glassware is therefore advisable. The presence of dust gave somewhat high values but the amount of dust ordinarily present is not larger as the quantities tested here. Nevertheless it is recommended that for accurate measurement the samples should be kept as free from dust as possible e.g the incubation should take place in closed tubes. Chlorhexidine gluconate which is a much used growth-inhibiting medium, precipitates in salt solution, and high concentrations give an opaque precipitate with the reagent solution, which makes spectrometry impossible. If the concentration is not higher than 0.002 W V there is, however no significant interference

Glass tubes are always used for incubation, since it was also found that the polyethylene tubes, which were initially used, adsorbed the chromogen.

Reproducibility

In testing the reproducibility the sources of error described above were, as far as possible avoided. Twenty five samples of a 30 μM/l glucose solution were determined by the same individual. All pipetting was done with an automatic syringe pipette (Serimatic®) in which the piston moves between two fixed (adjustable) points The syringe was of glass with a nylon delivery tip The coefficient of variation was = ? A calibration graph for the range 100—500 μM/l glucose did not deviate significantly from linearity

The calibration graph for the range 10—100 μM/l was not however quite linear (Fig 6

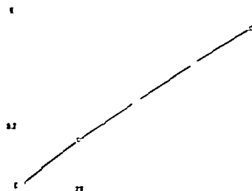


Fig. 6 Plot of absorbance against concentration of glucose The relationship is not quite linear within this range

Discussion

The clinical glucose determination method with Glox® involves two dilution procedures. First the blood is diluted with a protein precipitating reagent, and the supernatant is subsequently diluted with the glucose-oxidase reagent so that the final glucose concentration is about 50 μM/l. When non-protein containing solutions are used the precipitation step can be omitted, and this makes it possibl to deter mine glucose concentrations twenty times lower than in blood.

The enzyme concentration in the incubation tube was about the same in ll cases, i.e. at the recommended level (Hjelm and DeVerdier 1963). It should be noted that in these experiments the volumes of glucose solution were greater than with the recommended clinical method and therefore the stock Glox® solutions had to be of higher concentrations.

Of the sources of interference studied, light produced the greatest disturbance. E en illumination of short duration causes a marked decrease in absorbance which, however begins to increase immediately the sample is placed in the dark, and re turns to its original level after 20—30 min. In the method described the incubations were done in the dark in stoppered tubes which were kept in the dark until the bsorbance was measured in a spectrophotometer in a da kened room. It might be an ad ntage to use non-transparent incubation tubes.

Because of the interference of light on the reaction, t is ad isable to be careful in choice of photometer. The sample should not be illuminated with unfiltered light w th high intensity.

I wish to thank Mrs E. Engvist, Mrs H. Hogman, Mrs M. Waller and Miss E. Berglöf for aluable technical help. Financial support has been recei ed from the Medical Faculty, Uppsala U ersity and Swedish Society for Medical Research.

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Acta physiol. scand. 1967 71 194—202

From the Institut of Medical Physiology A, University of Copenhagen, Denmark

Inhibition of Salivary Secretion and Secretory Potentials by g-Strophantin, Dinitrophenol and Cyanide

By

Ole Holger Petersen and Jorgen Hedemark Poulsen

Received 17 April 1967

Abstract

Petersen O H. and J H. Poulsen *Inhibition of salivary secretion and secretory potential by g-strophantin dinitrophenol and cyanide* Acta physiol. scand 1967 [illegible] 194—202.

The hyperpolarization of the contraluminal acinar cell membrane which takes place when the submandibular gland is stimulated, is called the secretory potential Much indirect evidence has been presented in favour of the hypothesis that the mechanism of establishment of the potential is due to an active electrogenic chloride transport, and not to [illegible] change of the membrane permeability for ions This transport should be responsible for the formation of the saliva If this is true both secretory potentials and secretion should be inhibited to the same degree by metabolic inhibitors. In the present work it was found that the metabolic inhibitors dinitrophenol and cyanide and the cardiac glucoside g-strophantin (ouabain) had the ability to inhibit completely both secretion and secretory potentials in the perfused cat submandibular gland

Lundberg 1958 proposed that the secretory potentials in the acinar cells were due to an electrogenic transport of chloride, which should be responsible for the formation of the primary secretion

An [illegible] of the secretory potentials by inhibitors known to be able to impair active tr[illegible]port processes would be an important argument for the active nature of these [illegible] An inhibition of both secretion and secretory potentials by acetazol[illegible] which can inhibit active chloride transport in a number of organ[illegible]wn by Petersen and Poulsen 1966) However acetazolamide was onl[illegible] the [illegible] of the secretory potentials and the secretion, but not to abol[illegible] in a perfused gland to use potent inhibitors in concentrat[illegible] high to impair secretion completely In the present work on the perf[illegible]bmandibular gland it is shown that g-strophantin, dinitrophenol [illegible] have the ability of inhibiting both secretion and secretory potentia[illegible] the course of 5—10 min. This is a further support for the hypothes[illegible] processes are essential for the establishment of the secretory potential

Methods

Young cats were anesthetized with chloralose (70—80 mg/kg) given intraperitoneally. The preparation of the gland for artificial perfusion, the composition of the normal Locke solution and the recording of transmembrane potentials were described previously (Petersen and Poulsen 1967). In the present work the microelectrodes were filled with 3 M KCl.

Secretory potentials were elicited partly by electrical stimulation of the chorda-lingual nerve (10 V, 10 cps) and partly by intraarterial injection of 1 μg adrenaline or 5 μg acetylcholine (ACh). When ACh was used the number of drops of saliva evoked by the injection was counted. In this way the secretory ability of the gland and the secretory potentials could be recorded at the same time. In some experiments measurements of secretory rate during continuous infusion of ACh were carried out as described by Petersen and Poulsen (1967). The inhibitors were added to the Locke solutions to obtain the following concentrations: g-strophantin 10^{-4}M, 2,4-dinitrophenol 10^{-4}M, sodium cyanide $2 \cdot 10^{-3}$M.

Results

All the potentials in the present work belong to Lundberg's type I group, originating from the acinar cells.

The effect of g-strophantin (ouabain)

The inhibition of the secretory response to ACh is shown in Fig. 1. It is seen that already 1 min after the beginning of the g-strophantin perfusion, secretion is markedly impaired and after 8 min it is completely abolished. In Fig. 2 examples of normal secretory potentials and an example of a gradual inhibition of secretory potentials in the first period of the g-strophantin perfusion are shown. Fig. 3 shows the correlation between the inhibition of secretion and secretory potentials in one experiment. In Fig. 4 secretory potentials recorded during g-strophantin perfusion, after secretion had been reduced to less than 25 per cent of the control value are compared with secretory potentials from the control period. The majority of the secretory potentials in the group from 0 to 5 mV had a value of 0 mV corresponding to the fact that most of these potentials were recorded after secretion had stopped. The results were pooled from 4 expts. on 4 cats. The mean value of resting potentials in the control period was —19 mV (29 measurements). In the g-strophantin period the mean value was —12 mV (34 measurements).

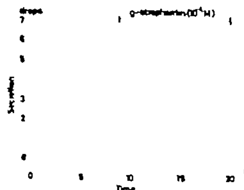


Fig. 1. Inhibition of secretion by g-strophantin (ouabain). Each circle represents the number of drops of saliva secreted after injection of 5 μg ACh.

Fig. 2. Examples of secretory potentials. A) Control period. The impalement of the cell (represented by the sharp potential drop) is shown. Thereafter two secretory potentials are evoked, the first by chorda stimulation (the stimulus artifact indicates the period of stimulation), the other by intraarterial injection of ACh (↓). B) A secretory potential recorded 1 min after start of g-strophantin perfusion. C) Resting and secretory potential 1 min later. D) Resting potential 3 min after start of g-strophantin perfusion. Stimulation gave no hyperpolarization. B), C) and D) originate from the same experiment on the same gland.



Fig. 3. Inhibition of secretion and secretory potentials by g-strophantin in a typical experiment. ○ = drops of saliva (ACh stimulation); ◆ = secretory potential (ACh stimulation); × = secretory potential (chorda stimulation).

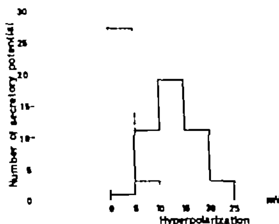
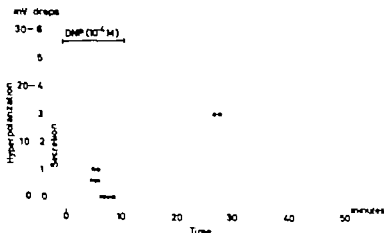


Fig. 4. Number of secretory potentials plotted as function of hyperpolarization. Two groups are shown, one ——— being control group of normal secretory potentials, the other - - - secretory potentials recorded during g-strophantin inhibition after the secretory rate had been reduced to less than 25 per cent of the control value. Secretory potentials were recorded partly after chorda stimulation and partly after ACh stimulation.

Th effect of dinitrophenol

The effect of dinitrophenol (DNP) in a typical experiment is shown in Fig 5 Both secretion and secretory potentials are abolished after 8 min. When a normal Locke s solution was reintroduced, it was possible to get some restitution of both secretion and secretory potentials. In the period f restitution the potentials elicited by nervous stimulation were somewhat smaller than the ACh-evoked potentials. This finding may be explained by a delayed inhibitory effect of DNP on the synapses between pre and postganglionic parasympathetic fibers. These synapses are located in the gland and are therefore perfused together with th gland. However during the perfusion with DNP there seems to be no discrepancy between the size of the secretory potentials elicited by nervous stimulation and those evoked by ACh. In Fig 6 secretory potentials recorded before inhibition, during DNP inhibition and



F 5 Inhibition of secretion nd secretory poten ial by DNP followed by restitution f er rors 1 of the inhibi or Symbol in Fig 3

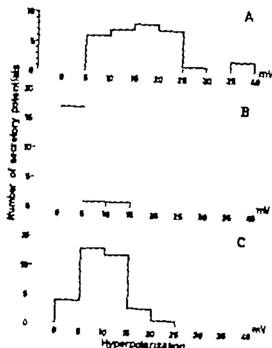


Fig. 6. Number of secretory potentials plotted as a function of hyperpolarization. A) Control group recorded before inhibition. B) Secretory potentials recorded during the DNP-inhibition after secretion had been reduced to less than 25 per cent of the control value. C) Secretory potentials recorded in the period of restitution after secretion had been restored to at least 50 per cent of the control value. Secretory potentials were recorded partly after chorda stimulation and partly after ACh stimulation.

in the period of restitution are shown. The potentials from the period of inhibition were recorded after the secretion had been reduced to less than 25 per cent of the control value but the majority were recorded after secretion had stopped and had a value of 0 mV. Secretory potentials from the period of restitution were recorded after secretion had been restored to at least 50 per cent of the control value. The

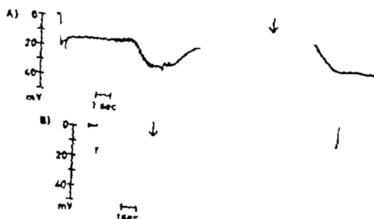


Fig. 7. Examples of secretory potentials. A) Control period. Impalement of the cell (represented by the sharp potential drop) is shown, and thereafter two secretory potentials are evoked, the first by chorda stimulation, the second by injection of adrenaline ( ). B) Resting potential recorded min after start of cyanide perfusion. Stimulation with adrenaline gave no hyperpolarization. A and B originate from the same experiment on the same gland.

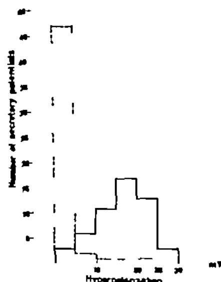


Fig. 8. Number of secretory potentials plotted as function of hyperpolarization. Two groups are shown, one being the control group of normal secretory potentials ——— the other secretory potentials recorded after secretion had been reduced to less than 25 per cent of the control value by cyanide – – – Secretory potentials were recorded partly after chorda stimulation and partly after adrenaline stimulation.

results were pooled from 5 expts. on 5 cats. The resting potentials in the three periods studied had the following mean values control period —18 mV (20 measurements) DNP period —16 mV (14 measurements) restitution period —17 mV (20 measurements)

The effect of cyanide

Secretion was generally abolished 2—3 min after introduction of cyanide. In Fig. 7 examples of normal resting and secretory potentials and an example of a resting potential which could not be raised by stimulation, as it was recorded in the period of complete cyanide inhibition, are shown. In Fig. 8 secretory potentials, recorded during cyanide perfusion after secretion had been reduced to less than 25 per cent of the control value are compared with secretory potentials from the control period. The majority of the secretory potentials from the cyanide period were recorded after the secretion had stopped and had a value of 0 mV The results were pooled from 5 expts. on 5 cats. The mean value of the resting potentials was —19 mV (40 measurements) in the control period and —15 mV (50 measurements) in the cyanide period.

Discussion

Ludwig (1851) showed that the processes underlying the formation of saliva are active transport processes. Before the investigations of Lundberg (1955) it was however impossible to state anything about the mechanism of these processes. With the detection of the secretory potentials and the investigations on the nature of these potentials, carried out in Lundberg's (1955 1957 a, b, c) pioneering work,

it was possible to advance the hypothesis of an active transport of chloride ions being responsible for the formation of the primary secretion and for the establishment of the secretory potentials

The active nature of the secretory potential was, however, seriously questioned by Imai (1965) who postulated that these potentials were due to an increased permeability of the contraluminal acinar cell membrane to potassium ions in the stimulated gland. This conclusion was based on perfusion experiments on the dog submandibular gland using varying potassium concentrations, where it was possible to abolish the secretory potential at high potassium concentrations while some secretion still remained. However, the problem was reinvestigated by Petersen and Poulsen (1967) who found in the perfused cat submandibular gland, that the size of the secretory potential and the secretory rate depended in very much the same way on the extracellular potassium concentration.

The results of the present work with different inhibitors of active transport are also very difficult to explain using Imai's model for the establishment of the secretory potential. The time course of the inhibition and the degree of the inhibition of both secretory potential and secretion was very similar. The theoretical possibility that all three very different inhibitors should inhibit an enhanced potassium permeability in very much the same way as the secretion, seems unlikely. Hodgkin and Keynes (1955) showed that DNP and cyanide although abolishing the active extrusion of sodium from the squid axon, did neither affect the resting membrane potential, which is mainly a potassium equilibrium potential, nor the electrical excitability of the membrane. The most likely explanation of the present results is that both secretion and secretory potential are due to an active ion transport across the contraluminal cell membrane.

Active transport of chloride ions has been demonstrated in several ion transporting systems. Hogben (19..) proved that the transport of chloride ions from the serosa to the mucosal side of the isolated gastric mucosa is an active one ... This transport ... inhibited ... by ouabain ... 10 M ... Cooperstein (19..) and by acetazolamide ... [illegible]

... Zadunaisky (1966) found that chloride is actively transported ... This transport was shown to be sensitive to g-strophanthin 10 M ... and to methazolamide ... analogue of acetazolamide ... 10 ... K ... and Hogben 1967.

Active chloride ... transport ... essential step in the formation of the primary secretion ... ed in the ... gland, where acetazolamide also is able to inhibit ... Enzl and Ullrich 19..

The finding ... submandibular gland that both secretion and secretory potential are ... g-strophantin as shown in this work and by acetazolamide as shown by Petersen and Poulsen 1966 fit very well with Lundberg's hypothesis of an active chloride ... the source of the formation of saliva and secretory potentials.

It should, however ... noticed that Lundberg's hypothesis for the formation of

the primary secretion has been based on studies on the cat sublingual gland. The concentrations of ions in the primary secretion are probably somewhat different in the submandibular and the sublingual glands. The primary secretion in the rat submandibular gland has the following ionic composition chloride 121 mmoles/l sodium 135—146 mmoles/l and potassium 6 mmoles/l (Martinez, Holzgreve and Frick 1966 Young and Schögel 1966) It is therefore probable that the bicarbonate concentration is about 20—30 mmoles/l in the submandibular primary secretion. In the dog sublingual gland there seems to be much less bicarbonate (Yoshimura *et al* 1959) which probably means that also in the primary secretion the bicarbonate concentration is small. The chloride concentration in the cat sublingual saliva is about 161 mmoles/l (Lundberg 1957 b) In the submandibular gland it is therefore likely that part of the active anion transport across the contraluminal acinar cell membrane is an active bicarbonate transport. An active transport of bicarbonate ions has been discovered in the turtle bladder which also actively transports chloride and sodium (Schilb and Brodsky 1966)

In the present work the mean resting potential in the acinar cells of the artificially perfused cat submandibular gland was found to be —19 mV In other investigations values of —17 and —18 mV have been found (Petersen and Poulsen 1967 Petersen, Poulsen and Thorn 1967) In the autoperfused gland the resting potentials seem to be somewhat higher —22 mV (Lundberg 1955) and —21 mV (Poulsen and Petersen 1966) It is possible that the lower temperature (room temperature) in the artificially perfused glands has influenced the potentials. The question about the nature of the resting potentials must still be left unanswered. It is dependent on the extracellular potassium concentration in very much the same way as the size of the secretory potential and the secretion (Petersen and Poulsen 1967) and decreases by removal of calcium (Petersen *et al.* 1967) As shown in the present work cyanide and g-strophantin depress the resting potentials somewhat while DNP does not affect the resting potentials significantly

The authors are greatly indebted to Prof N A Thorn for his valuable help and criticism throughout this study

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p tential Much evidence has been presented in favour of the hypothesis that the secret ry potential is due to an acti e tr n port of chloride ions from the extracellular pace through the contraluminal acinar cell membrane into the cell. This transport is supposed to be the energy demanding part of tl salt and w ter transport which results in the formation of the primary secreti n Lundberg 1958, Petersen and I ulsen 1966 1967 a b

In order to try to find an explanat on of Dou las and I oimer 1963 esults, the eff ct of remov ng calcium fr m the perfusion fluid on the secretory potentials and the water permeability of the duct system wa investigated. It was found that the size of the secretory potentials *remained unaltered* while the *hape changed markedly*. The w thdrawal of calcium did not chang th water permeabilit of th duct system in the unstimulated gland

Methods

Cats weighing 1—4 kg were anesthetized ith hloralose 0 50 mg g intraperitoneall . The preparation of the gland and duc the prepar tion for artificial perfusion and he methods for measurement of the tr nsmembrane potentials ha previously been desc bed Petersen and Poulsen 1 7 . In the present work the microelectrodes ere filled with ither 3 M KCl or 3 M NH NO. The perfusion fluid used as Lo ke solution ith he follow [illegible] mmoles l Na 142, K 4.0 Mg 1.0 Cl 12 HCO 22, HPO 1.1 gluco [illegible] as either present in concen tion of 1 mmoles l or was completel omitted. I [illegible] wi h calcium-free Locke sol tion EDTA eth lenediaminetetra-acetic acid in [illegible] of 10 M was present. The perfusion fluid was equilibrated with —6 CO in

Stimulation of the gland was al arried out by lose [illegible] injection of acet lcholine ACh g in olume of 50 µl. Both the secr [illegible] has drops of sali secr ed, and the secretory po ential was measured [illegible] h,

The possibility that the perme bility water [illegible] unstimulated gland hanged after perfusion with the calcium-free [illegible] in 3 perimenti which tritium labelled w ter THO dure [illegible] injected retrograde from the duc to du [illegible] with alcium-free Locke sol tion. The perf [illegible] periods and the radioactiv of the injection [illegible] liquid scintillation counter

All the secret ry potentials in the p gr up originating from the acinar cell

F g 1 is a schemat cal draw ng of a re parameters stud ed in this w rk are sho

An experimer t with calcium-free perfu the secret ry esponse to ACh gradually sizes of the secretory potentials measured unchanged. In Fig 3 the distribution of the during calcium free perfusion are c recorded with normal calcium concentratio por ed from 17 expts. on 17 cats. It is e 1 examples from the tw gro

potential. Much evidence ha been presented in favour of the hypothesis that the secretory potential is due to an active tran port of chloride ions from the extracellular space through the contraluminal acinar cell membran into the cell. This transport is supposed to be the energy demanding part of the salt and water transport which result in the formation of the primary secreti n (Lundberg 1958, Petersen and Poulsen 1966, 1967 a, b).

In order to try to find an explanation of Dougla and Poisner's (1963) results, the effect of removing calcium from the perfusion fluid on the secretory potentials and the water permeability of the duct system was invest gated. It was found that the size of the secretory potentials remained unaltered, while the hape changed markedly. The withdrawal of calcium d d not change the water permeability of the duct system in the unstimulated gland.

Methods

Cat eighing 1—4 kg were anesthetized with hloralose (0 90 mg/kg in raperitoneally. The prepar tion of the gland nd it duct, the preparation for rt ficial perfusion nd the methods for measurement of he ra unembrane potential h previously been described (Petersen and Poulsen 1967. I he present w rk he microelectrodes were filled h either 3 M KCl or 3 M NH_4NO_3. The perfusion fl id used was a Lock sol ion w th the follow g composition (mmoles/l) : Na 150, K 4.0, Mg 1.0, Cl 127, HCO_3 25, HPO_4 1.1, glucose 10. Calcium as either present at oncent tion of 1 mmoles/l or w completely omitted. I some periments with calcium-free Locke solution EDTA (eth lenediaminetet a-acetic acid) in a oncen tion of 10^{-4} M as present. The perfusion fluid was equil bra ed w th 5—6 CO_2 in O_2.

Stimula ion of the gland w alw y arried out by lose raarterial jection of acetylcholine (ACh) (5 μg in ol m of 50 μl). Both he secretory esponse measured as drops of sal secreted, nd h secretory potential as measured fter every injection of ACh.

The possibil hat he permeability t w ter of he duct ystem the unstimulated gland is hanged f ee perfusion w h he lcium-free Lock olution ested in 3 experiments, in which n um labelled w er THO (d luted with either 0.9 NaCl solution or ater) as injec ed retrogradel f om he duct in olumes of 25 100 μl, during perfusion h normal and with alci m-free Locke ol ion. The perfusion fluid as collected in 1 or ml fractions at stated period and he dio tiv of he jec ion fl id nd the perfusion fluid as measured in Packard liquid sc lla ion oun er.

Results

All the secr tory potential in the present work belong to Lundberg's (1955) type I group, orig nating f om the cinar cells.

Fig. 1 a schemati al drawing of a resting and a secretory potential. The d fferent parameter stud ed in th s work are shown.

An experiment t calc um free perfusion fluid is shown n Fig. 2. It is seen that the secretory respo se to ACh gradually diminished over a 50 m n period while the sizes of the sec t pot ntials measured after the sam ACh injections remained unchanged. In F g. 3 the di t ibution of the sizes f the secretory potentials measured during calcium f ee perfusion are compared with the sizes of secretory potentials recorded with normal calcium concentration in the perfusate. The data ha e been pooled from 17 expt on 17 cats. It is evident that no difference between the two groups exists. Typical examples from the two groups are shown in Fig. 4. The differ

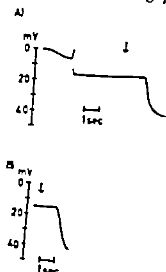


Fig 4 Tw typical secretory poten ials from each of th tw groups show in Fig 3 I A) secretory potent al from calc m-free period is shown. The actual mpalement of the cell is indicated by the harp pe ential drop. The secretory response t the i jection of ACh which resulted in the secretory potential w ero EDTA (10 M) was presen in he perfusion fluid. I B) normal secretory potential is how Th rrow represent injection of ACh (3 μg)

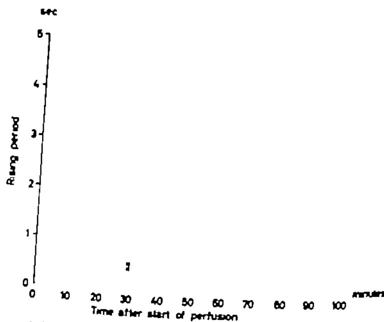


Fig 5 Risi g period as function of time after start of perfusion = alues from secretory potentials recorded during calcium-free perfusion after secretion had been reduced to less than 25 per cent of the control level X = values from control potentials.

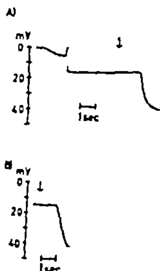


Fig. 4. Two typical secret ry pote tials from each of the t groups show in Fig. 3. I A) secretory potential from alc m-free period is shown. The actual impalement of the cell is indicated by the sharp potential drop. The secretory esponse to he jection of ACh which resulted in the secretory potential was zero. EDTA (10 M) was present in the perfusion fluid. I B) normal secretory potential is shown. The arrow epresent jection of ACh (5 µg)

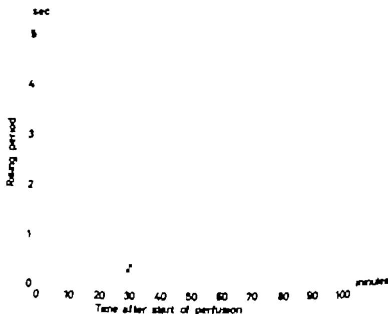


Fig. 5. Rising period as function of time after start of perfusion. — alues from secretory potentials recorded during calcium-free perfusion after secretion had been reduced t less than 25 per cent of the control level. × = values from control potentials.

ence in shape is characteristic. Fig 5 shows that there exists a marked difference between the duration of the rising period in the two groups of secretory potentials. Fig 6 shows that there is no striking difference in maximal hyperpolarization rate between the two groups.

The mean valu of resting potentials during perfusion with normal Locke's solution was 17 mV (35 measurements) During perfusion with calcium-free solution the mean value was —11 mV (19 measurements)

In the 3 expt in wh ch the permeability to THO of the duct system was tested, there seemed to be no difference between the rate of appearance of THO in the perfused solution in the experiments with normal Locke's solution and with calcium-free Locke's solution Nor was the total number of counts recovered during the first 12 collection periods after the injection of THO s gnificantly different In Fig. 7 are seen the result of an experiment in which 25 μl of THO in H_2O was injected retrogradely through the sali ary duct to a cat weighing 4 0 kg As can be seen the radioactivity pattern was very similar during perfusion with normal Locke calcium-free Locke and after restitution following perfusion with the calcium-free Locke. The amount f sal a secreted following injection of ACh before and after each of the 3 experimental periods is shown.

Discussion

According to Lundberg 1957 the formation of the primary secretion is the result of th f llow ng processes The first tep is an active electrogenic transport of chloride ion thr ugh the contraluminal acinar cell membrane into the cell As a consequence of thi mechanism, which increases the intracellular negativity cations (mainly sodium e passi ly transported into the cell This will crease the intracellular osmolarit C nsequentl water diffuses into the cell thereby increasing the intracellular pressure Thi causes a filtration through the luminal cell membrane resulting n the formation of the primary secretion (but also a filtration through the contraluminal membrane

Ther are several p ssibl explanations of the fact that sal vary secretion stops in the absen e of al um

1 Th c nd ti ns n tl ac ni are normal but in the duct system an increased water permeabili makes water follow sodium, which is actively reabsorbed in the duct th p mary secretion formed in the acini is completely reabsorbed in the duct t m

2) The t ansc ll l ter t ansport across the acinar cells s normal, but the primary secretio es p th ough leaky ntercellular spaces.

3) The transport l salt nd water into the cells across the contraluminal acinar cell membrane s al but the water permeability of this membrane is increased Therefore all fluid f lt d back through this membrane

4) The transport of chl de and sodium ions nto the cinar cells is unpaired.

ad 1) Since no cha g n th permeability of the duct sy tem to THO after removal of calcium could be detected th possibility does not seem likely

ad 2) It has been shown (Sedar and Forte 1964) that treatment w th EDTA dissolved the junctional complex between oxyntic cells in the gastric mucosa of the frog This effect was reversible on readministration of calcium. It does not seem unlikely that a similar phenomenon could take place in salivary glands. However it is impossible to say whether the whole amount of primarily secreted fluid could escape through such leaks.

ad 3) This is a theoretical possibility Lamiter et al (1965) showed that the water permeability of the distal rat kidney tubule increased in the absence of calcium.

ad 4) At the first sight the finding that the sizes of the secretory potentials were unaltered after removal of calcium seems to exclude this possibility It should be noted however that the size of the secretory potential depends partly on the rate of active transfer of chloride ions across the contraluminal acinar cell membrane and partly on the ease with which cations (mainly sodium) passively follow the chloride ions, i.e. the conductivity of the cell membrane to sodium. If for instance the sodium conductivity is very low in calcium-free medium, a very low transfer rate of chloride ions could still give a secretory potential of a normal size. In the squid axon Frankenhaeuser and Hodgkin (1957) showed that the passive, but probably carrier mediated sodium transport into the axon, which is responsible for the action potential, is inactivated in the absence of calcium. It should be noticed that the shape of the secretory potentials was definitely changed (shown by the marked increase in rising period) when calcium was removed. The significance of this finding is obscure, but it might indicate that the balance" between active chloride transport and secondary sodium influx is reached at a changed (reduced) transfer rate of chloride

The results of th present work throw some light on the role of calcium in the so-called stimulus-secretion coupling

McCarthy and Sheehan (1966) showed that calcium was necessary for the release of ACh from the nerve endings t the gland because the secretory response to chorda timulation was abolished much sooner than the response to njected ACh after remo-al of calcium. The results of the present work show that the acinar cells react on ACh jection even in th period where secretion is completely abolished, 50—60 min after removal of calcium, as secretory potentials can still be elicited. This can be ompared w th the findings of Douglas, Kanno and Sampson (1966) who were able t show depolarizing effect of acetylcholine and other medullary secretagogues on isolated cells from the drenal medulla. This depolarizing effect of cetylcholine persisted n a calcium-free medium. Correspondingly Harvey and MacIntosh (1940) found in the perfused superior cerv cal ganglion of the cat that synaptic transmission failed when calcium was omitted from the perfusion fluid This failure was due to failure of preganglionic impulses t liberat ACh It was shown that postsynaptic ganglion cells were still ble to react on nject ons of ACh In some hormone releasing stems (adrenal medulla, nerve endi gs and posterior pituitary gland) calcium enters the cell during timulation and p bably releases hormone by a simple bio-hemical mechanism Thorn 1965 It would ppear that the role of calcium in the secretion of salt and w ter from salivary glands may be only to secure normal

permeability conditions in the glands. It is possible however that an intracellular transmitter function of calcium as it occurs in the above mentioned hormone releasing systems, is required for the secretion of proteins from salivary glands. In the experiments of Douglas and Poisner (1963) there was a marked reduction in the secretion of protein when calcium was omitted from the perfusion fluid. This can be compared with the finding of Hokin (1966) that the ACh stimulated release of enzymes from pancreas [illegible]lices requires the presence of calcium.

This work w[illegible] supported by a grant from the Danish State Research Foundation.

Addendum:

I[illegible] recent experiment[illegible] it ha[illegible] been shown that when [illegible]ll [illegible]hloride ions in the perfusion fluid are substituted by sulpha[illegible] ions, i[illegible] is [illegible]till possible to record normal secretory potentials, [illegible]hile the ability of the gland to secret[illegible] is [illegible]ompletel[illegible] ab[illegible]ished. It would therefore [illegible]ppe[illegible] that the secretory potential may not be due to an [illegible]ctiv[illegible] transport of [illegible]hloride ions, but that it may be due to some other ion transport. If [illegible]his is true[illegible] it is possible that calcium play[illegible] [illegible] more direct role in the mechanism of secretion. The experiments w[illegible]th sulphate-containing chloride-free perfusion fluid [illegible]ll be described in [illegible] subsequen[illegible] paper.

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Further Studies on the Effect of Follicle Stimulating Hormone on Amino Acid Transport in the Isolated Rat Ovary

By

KURT AHRÉN, LARS HAMBERGER and MARIANNE HARTFORD

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Abstract

AHRÉN, K., L. HAMBERGER and M. HARTFORD. *F rther studies n the effect of follicle stimulati g h rmone amino acid t ansport i th isolated at ovary.* Acta physiol. scand. 1967. *71*. 211—223.

Amino acid ptake by isolated whole ovaries from prepuberal rats has been studied employing the non-utilizable amino acids α-amino-isobutyric acid (AIB) and 1-aminocyclopentane carboxylic acid (cycloleucine) as well as the normal amino acid proline. The ovaries showed nearly linear uptake of AIB during 4 hrs of incubation while cycloleucine reached steady-state levels in the tissue after approximately 1 hr. The uptake of proline resembled that of cycloleucine. Ovaries from rats which had received an i. injection of FSH before the extirpation of the ovaries showed both higher rate of AIB-uptak and higher levels in the steady-state distribution of cycloleucine than ovaries from control rats. Dose-response rela tionships as well as the importance of the time interval between the FSH injection and the start of incubation were analysed. Addition of FSH directly to the incubation medium had no stimulatory effect on amino acid uptake. I traperitoneal injection of puromycin before the injection of FSH blocked the effect of the hormone on AIB-uptake. Various possibilities to explain the lack of *in* *ure* effect of FSH on amino acid uptake are discussed in relation to the observations with puromycin.

In 1963 Ahrén and Kostyo reported that an i. injection of follicle stimulating hormone (FSH) 4 hrs before extirpation of the ovaries from prepuberal rats, stimulated the intracellular ccumulation *in vitro* of the non-utilizable amino acid α-aminoisobutyric acid (AIB). Addition of FSH directly to the incubation medium did not ha this effect. In subsequent experiments, Ahrén and Rubinstein (1965) found that ovaries from FSH-injected rats showed a higher concentrative uptak not only of the amino acid analogue AIB but also of glycine and aline. The relationship between ncorporation of normal amino acids into ovarian protein nd the ac cumulation of amino cids in the cell water was also studied. It w found that the increased ptake of amino acids in the ovaries from FSH-injected t ld not be explained as an effect solely secondary to timulation of amino acid in rporation into protein. It was therefore concluded that the effect of FSH n amino acid transport might represent primary action of this hormone.

The nature of this acut effect of FSH on the rat ovary has now been further explored and results concerning the following aspects are reported in the present paper 1) kinetics of the uptake of two non-utilizable amino acids, AIB and 1-amino-cyclopentane carboxylic acid (cycloleucine) and of the normal amino acid proline 2) variation in the time interval between the FSH injection and the start of incubat'on 3) dose response relationships 4) influences of puromycin on the FSH effect

Methods

A mals

Female rats, 4 ?o day old of the Sprague-Dawley strain were used. I l expt hypophysectomized rats were sed The completeness of hypophysectomy was confirmed at utopsy by serial section through the hypophyseal capsule and adja ent tissues, including the pituitary stalk and media eminence The rats were given semisynthetic diet (Gustafsson 1959) nd water *d lib t m* Non hypophysectomized rats were deprived of food ?0— 4 hrs before dissection

Chemic l

AIB-1 ${}^{4}C$ and generally labelled L-proline- H were obtained from the Radiochemical Centre Amersham England Cy loleucine-1 ${}^{14}C$ was obtained from New England Nuclear Boston, M ss U S A The substances were used with the following specific ctivities AIB- ${}^{4}C$ 3 0 µC µmole when the medium concentration w 0 1 mM and 16 3 µC µmole when the concentra ion w 0 1 mM (see Result) cycloleucine ${}^{14}C$ 1 8, µC µmole proline-${}^{3}H$, 100 µC mole

Puromyci dih drochloride w obtained from N tional Biochemicals Corp Cleveland, U S A

Ovine FSH NIH FSH S2) wa supplied by the Endocrinology S udy Section of the N tional Inst t tes of He l h, U S A The hormone was dissolved in 0 9 N Cl (1 mg ml) for the injec ion and in Krebs bicarbonate buffer when dded in *it*

P moc l d bation f ov ie

The rat were killed by cerv l fra ture The ovaries were rapidly removed, trimmed of l sue and ext aneous ssue under dissecting microscope nd incubated in Krebs bicarbonat buffer con aining 5 mM glucose plus the arious labelled substances as described in previous pape Ahren nd Rubinstein 1965) This study differed from hose described in ha only two ov ries were incuba ed in each flask (10 ml Erlenmeyer flasks) The flasks w re g ssed w h 9 O 5 CO_2 and incubated at 37 C for arious periods with continuou sh k ng F llowing incubation the flask wer placed on ice nd the media were withd w mmedia ly Th ovaries were removed from the flasks, rinsed free of adhering incuba ion med m by pid wash in ice-cold buffer blotted on filter paper weighed immediat l nd hen homogenized in 1 ml 10 trichloroacetic acid TCA)

M as m d al latio

The radio c n f lu ion media and TCA extracts wa assayed in Packard Tri-Carb liquid scin illa ior spec rome described in previous paper (Ahrén and Rubinstein 1965)

Determina ons f l and xt ac llular tissue w ter content under arious experimental conditions w ll me surem ts nd alculations of the intracellula accumulations of AIB- ${}^{4}C$, cy lolcu (nd proline- H were determined as described previous paper (Ahrén and R bi st 1 6 Th int ac llular accumulations of these substances are expressed as distribution tios pm ml intracellular water- cpm ml incubation medium) hich indicate the exte h h h mino acid was concentrated in the ell water Chromatographic analyses of he sue ex acts showed ha AIB- ${}^{4}C$ and cycloleucine- ${}^{4}C$ were not metabolized by th ov ria issue The amino acid proline can of course be metabolized to some extent in the ovaria ll nd the experiment with proline- H it is therefore likely that some of the radioa y in the issue extracts did not represent the original amino acid, but various metabolites This w not nalysed in the present experiments. It is therefore important to point out hat d tribu ion ratios for this amino cid are the ratios between the radioactivity in the total tissu w nd the radioactivity in the incubation medium

Lactic acid was determined by an enzymatic method described by Lundholm *et. al.* (1963 *a, b*) and the production of lactic acid by the isolated ovaries during the incubation period was calculated as µg/100 mg wet tissue weight as described in detail in previous paper (Hamberger and Ahrén 1967)

Statistical analysis

Mean values are given ± standard error of the mean. Comparison between different groups was performed according to Student' t-test. A p-value of 0.05 or less was considered significant in this study Calculations of regression line correlation coefficient and test of linearity were performed according to Brownlee (1965)

Results

A. Kinetics of the amino acid uptake

Fig 1 shows the concentrative uptake of AIB under 4 hrs of incubation by isolated prepubertal ovaries from control rats and from rats injected with FSH (500 µg/100 g b.w) 2 hrs before the start of incubation. It is seen that the intracellular concentration of AIB continued to increase at a nearly linear rate for the whole incubation period, and that there was no sign of leveling off to a steady-state plateau. From the curve it seems to be an initial slower phase lasting for about 30 min followed by a more rapid linear rate This trend with two phases was most marked in the ovaries from the FSH-injected rats. Fig 1 also shows that there was a clear difference in the rate of AIB-uptake between the ovaries from control and FSH-injected rats. A difference in distribution ration measured after 2 or 4 hrs of incubation seems to be a good indication of a true difference in the rate of accumulation in studies with this amino acid analogue.

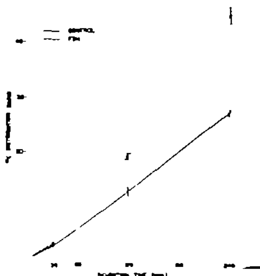


Fig 1 Accumulation of AIB by isolated ovaries from prepuberal rats FSH was injected 2 (500 µg 100 g b w) hrs prior to incubation The ovaries were incubated in Krebs bicarbonate buffer pH 7 4 containing 0 01 mM AIB-¹⁴C and 5 5 mM glucose Each point represents the mean of three observations and the standard errors are indicated by vertical lines I the absence of such lines, the standard error was too small to be indicated The difference between ovaries from control and FSH injected rats was significant already after 30 min of incubation (controls 2 5±0 1 and FSH 3 0 0 1 P<0 05)

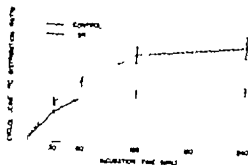


Fig. 2

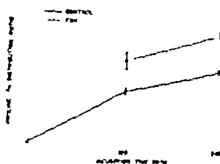


Fig. 3

Fig. Ac umulation of cy loleucine-^{14}C by isolated ovaries from prepuberal rats. FSH was injected 1 (500 µg 100 g b.w.) hrs prior t incub tion. The ov ries were incubated in Krebs bicarbonat buffer pH 7.4 containing 0.1 mM cy loleucin and 5.5 mM glucose. Each point represent the me n of 3 observ ions nd the standard errors are indicated by vertical lines. The difference between ovaries from control and FSH injected rats became s gnificant after 60 mi of incubation (P<0.05 or less).

Fig. 3. Accumulation of proline-^{3}H in the intracell lar water of isolated ov ies from prepuberal rats. FSH was ected i (500 µg/100 g b.w.) hrs prior to incubation. The ovaries ere incubated in Krebs-Bica bonate buffer pH 7.4 containing 0.1 mM proline-^{3}H and 5.5 mM glucose. E h point represents the me of 4 observ tion nd the standard errors are indicated by ertical lines. The differences between ovaries from saline and FSH-injected rats are significant (P<0.05 ft hrs of incubation P<0.001 after 3 hrs).

The uptake data relating to the other non-utilizable amino cid cycloleucine and to proline are shown in Fig. 2 and 3. It can be seen from F g. 2 that the ovaries initially took up cycloleucine more rapidly than AIB but that the distribution ratio for cycloleuc ne came to a steady state after about one hr of incubation. The ovaries from the FSH injected rat showed both a more rapid initial rate of uptake and a higher level of the steady state distribution than the corresponding control ovaries. Fig. 3 demonstrates that the distribution data for proline resembled those of cycloleuc ne more than those of AIB. A clear effect of FSH wa seen also on the distributions of prolin

B l art t with th dose of FSH and with the injection h dule

In one series of experiments three doses f FSH (5 50 and 500 µg 100 g b.w.) were injected to prepuberal rats 2 4 and 9 hrs, respectively prior to removal of the ovaries. The cont l rats ere njected with saline. All injections ere given under light ethe esthesia. The ovaries were incubated i bi arbonate buffer containing 5.5 mM gluco nd 0.01 mM AIB-^{14}C and the AIB distribution ratios were determined after 2 h of incubation. It can be seen from T bl I that the highest dos of FSH (500 µg 100 g b.w.) timulated the AIB- ptak when injected 2 or 4 hrs prior to incubation while there was no effect hen the time nt rval was 9 hrs. It can also be seen that the effect was most pronounced hen the hormone was given 2 hrs before incubation nd that the two smaller doses (50 and 5 µg/100 g b.w.) were also effective under this condition. Fig. 4 shows that there was a log linear dose

Table I. Effects of various doses of FSH injected 2, 4 and 9 hrs prior to incubation on the AIB-uptake by isolated rat ovaries[a]

Time interval hrs	Dose of FSH µg/100 g b.w	AIB-14C distribution ratio[b]	Significance of FSH effect
2	0	10.9±0.3 (12)	
2	5	13.5±0.6 (7)	P<0.005
2	50	14.6±0.6 (7)	P<0.001
2	500	17.1±0.5 (7)	P<0.001
4	0	11.0±0.7 (4)	
4	5	10.4±0.3 (4)	N.S.
4	50	11.7±0.8 (4)	N.S.
4	500	15.5±0.9 (4)	P<0.01
9	0	10.4±0.7 (5)	
9	5	10.2±1.0 (4)	N.S.
9	50	9.8±0.2 (4)	N.S.
9	500	9.3±0.9 (4)	N.S.

[a] FSH was injected to prepuberal rats in one [illegible] dose under light ether anaesthesia. The controls were injected with saline. The ovaries were incubated for 2 hrs in Krebs bicarbonate buffer containing 0.01 mM AIB-14C and 5.5 mM glucose.

[b] Ratio of cpm/ml intracellular water : cpm/ml medium. Mean ± S.E. Number of observations in parenthesis (N.S. = not significant)

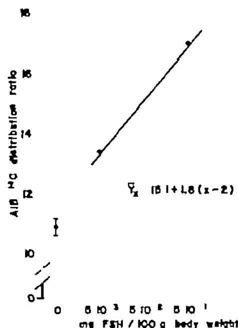


Fig. 4. The regression line $\hat{Y}$ of the AIB-14C distribution ratios in isolated ovaries from prepuberal rats injected with 3 different doses (5, 50 or 500 µg/100 g b.w.) of FSH 2 hrs before the start of incubation. The ovaries were incubated for 2 hrs in Krebs bicarbonate buffer pH 7.4 containing 0.01 mM AIB-14C and 5.5 mM glucose. Each point represents the mean of 7 observations. The line with the equation $\hat{Y}$ = 13.1 + 1.8 (x−2) is linear with a correlation coefficient of 0.70, which is highly significant (P [illegible] 0.001). The distribution ratio of ovaries from simultaneously incubated ovaries from control rats is indicated to the left in the figure (mean ± S.E.)

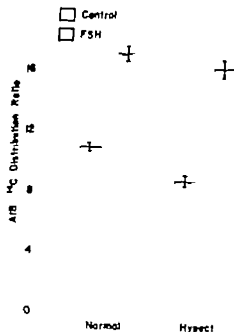


Fig. 5. Effects of FSH (500 μg/100 g b.w.) injected 2 hrs prior to incubation on the AIB-uptake in ovaries from normal prepuberal rats and from hypophysectomized rats. The ovaries were incubated for 2 hrs in Krebs bicarbonate buffer pH 7.4 containing 0.01 mM AIB-14C and 5.5 mM glucose. There were 3–4 observations in each group and standard error of the mean is indicated on the top of each column. The effect of FSH is highly significant in both groups of ovaries (P < 0.001).

TABLE II. Effect of FSH injected 30 min prior to incubation and effect of anesthesia on the AIB-uptake by isolated rat ovaries

Expt.	Type of anesthesia	Dose of FSH μg/100 g b.w.	AIB-14C distribution ratio[1]	
			2 hrs of incubation	4 hrs of incubation
I a	Ether	0	16.1 ± 1.4 (3)	—
b	Ether		17.4 ± 1.4 (3) N.S.	—
c	Ether	50	16.2 ± 0.5 (3) N.S.	—
d	Ether	500	18.5 ± 0.5 (3) N.S.	—
II a		0	18.6 ± 1.1 (4)	33.7 ± 1.3 (3)
b		500	19.1 ± 0.5 (4) N.S.	36.0 ± 2.5 (3) N.S.
III a	Nembutal	0		23.1 ± 1.5 (3)
b	Nembutal	500	—	29.5 ± 1.7 (3) P < 0.05

FSH was injected to prepuberal rats in one dose. Ether anesthesia was given for ~3 min. Nembutal was given in a dose of 4 mg/100 g b.w. 15–30 min before the injection of FSH or saline. The ovaries were incubated for 2 or 4 hrs in Krebs bicarbonate buffer pH 7.4 containing 0.01 mM AIB-14C and 5.5 mM glucose.

[1] Ratio of cpm/ml intracellular water to cpm/ml medium. Mean ± S.E. Number of observations in parenthesis. The significance of the FSH effect is indicated for each group and comparisons were then made with the distribution ratio of the appropriate control group.

relationship between the three dose levels of FSH and the AIB distributions after 2 hrs of incubation when the hormone was injected 2 hrs before the start of incubation.

In 1 expt. FSH was injected 2 hrs before the start of incubation in a dose of 500 µg/100 g b.w. to hypophysectomized rats. These rats were operated on when 28—30 day old and used for the experiments 7 days later. The two ovaries from each of these hypophysectomized rats weighed 5.4—9.6 mg. The corresponding weight of ovaries from the 24—26 days old normal prepuberal rats which were used in all the other experiments of the present study was 10—15 mg. Fig. 5 shows that this dose of FSH had as pronounced effect on the ovaries of the hypophysectomized rats as on the ovaries of the normal prepuberal animals.

In other experiments (Table II, part I) FSH was injected to normal prepuberal rats, 30 min before the start of the incubation period, and AIB distribution ratios were measured after 2 hrs of incubation. It can be seen from Table II that there was no effect by any of the FSH doses used, but it can also be seen that the distribution ratios of the ovaries from the control rats (i.e. rats given an i.v. injection of saline under light ether anesthesia) was much higher than in other experiments (comp. Table I). The nature of this influence of ether anesthesia is not clear and ought to be further analysed. It can, however, be concluded at present, that ether anesthesia so close to the start of the incubation period is not suitable in this type of experiment.

When the rats were given the i.v. injection in the tail vein 30 min before the start of incubation without any anesthesia, it was found that this procedure also influenced the rate of AIB-uptake in vitro (Table II, expt. II). Experiments were therefore performed with intraperitoneal (i.p.) nembutal anesthesia. This did not in itself influence the rate of AIB-uptake under a subsequent incubation period even though the rats were kept under anesthesia until they were killed. An FSH injection (500 µg/100 g b.w.) 30 min before the start of incubation stimulated under this condition the AIB-uptake in vitro (Table II, expt. III). The effect was less pronounced than when the time interval was 2 hrs. The experiments were therefore performed only with the higher dose of FSH.

Table III shows that an injection of FSH (500 µg/100 g b.w.) 5—7 min before the start of incubation significantly stimulated the AIB-uptake in vitro. These experiments were performed with nembutal anesthesia and the rats were anesthetized for 20—25 min before the injection. Control ovaries of nembutal anesthetized rats were also incubated with the addition of various concentrations of FSH (up to 500 µg/ml) to the incubation medium, but no effect of FSH in vitro on the AIB-uptake could be seen after 2 or 4 hrs of incubation.

Effect of puromycin on the effect of FSH

In previous experiments (Ahrén and Rubinstein 1965) it was found that when ovaries from prepuberal rats were incubated for [illegible] hrs the addition of puromycin dihydrochloride in a concentration of 500 µg/ml to the incubation medium com-

TABLE III Effect of FSH injected 5—7 m[illegible] prior t[illegible] incubation on the AIB-uptake by isolated rat ovaries

Incubation time hrs	AIB-[illegible]C distribution ratio		Significance of FSH effect
	Control	FSH	
2	10.5±0.3 (4)	13.4_0.6 (4)	P 0.01
4	19.8±1.1 (4)	27.3±1.3 (4)	P<0.005

FSH was [illegible]jected i[illegible] prepubertal r[illegible]ts in one [illegible] dose (500 μg/100 g b.w.) under nembutal anesthesia. Nembutal was gi[illegible]en [illegible]p. i[illegible] a dose of 4 mg/100 g b.w. 15—20 mi[illegible] before the injection of FSH or saline. Ovaries were incubated for 2 and 4 hrs i[illegible] Krebs bicarbonat[illegible] buffer pH 7.4 containing 0.1 mM AIB-[illegible]C and 5.5 mM glucose.

R[illegible]tio of cpm/ml [illegible]tracellular w[illegible]ter [illegible] cpm ml medi[illegible]m. Mea[illegible] ±S.E. N[illegible]mber of observations in parenthesis.

pletely blocked the incorporation of labelled glycine-^{3}H into the ovarian protein. It was also found that the rate of AIB-uptake was slower under this condition. However, also under this condition the ovaries from FSH injected rats took up AIB more rapidly than ovaries from the saline injected controls. The increased rate of amino acid uptake in ovaries from FSH injected rats could therefore not be explained solely as a phenomenon secondary to a concomitantly increased rate of amino acid incorporation into prot[illegible]in.

In the present study experiments were performed to explore whether treatment of the rat with puromycin could block the acute effect of FSH. Puromycin was injected i.p. in a dose of 5 mg 15 min before an i.v. injection of FSH (500 μg/100 g b.w.). Puromycin was again injected one hr afte[illegible] the FSH injection, and the ovaries were removed for incubation one hr later, i.e. 2 hrs after the FSH injection. T[illegible]o expts. were performed: one with addition of puromycin (500 μg/ml) to the incubation medium and one without this addition. The ovaries in both experiments were incubated for 2 hrs in Krebs bicarbonate buffer containing 0.1 mM AIB-[illegible]C and 5.5 mM glucose. In the second of these two expts. (Table I[illegible] groups 1—4) lactic acid production was measured in addition to the determination of AIB-uptake.

Table IV show[illegible] t[illegible]e AIB-distributions in these two experiments. It is clearly seen that FSH did n[illegible]t s[illegible]mulat[illegible] the AIB-uptake under a subseq[illegible]ent incubation period when the hormone w[illegible]s injected to puromycin treated rats. In the experiment, where la[illegible]tic acid prod[illegible]ction was measured it was found that the ovaries from the FSH-injected rats prod[illegible]c[illegible]d more lactic acid under the incubation period than the ovaries from the control rats, and that this effect was the same also when the hormone was injected to puromycin treated rats.

In another experiment, FSH (500 μg/100 g b.w.) was injected t[illegible] prepuberal rats 30 min before the start of incubation. These rats were anesthetized with nembutal

TABLE IV. Influence of puromycin on the effect of FSH on the AIB-uptake by prepuberal rat ovaries[a]

Group	FSH in vivo	Puromycin in vivo	Puromycin in vitro	AIB-^{14}C distribution ratio[b]	Significance of FSH effect
1	—	—	—	12.9±0.4 (4)	P<0.005
2	+	—	—	17.7±0.7 (4)	
3	—	+	—	13.1±1.4 (3)	N.S.
4	+	+	—	13.8±0.8 (3)	
5	—	—	+	9.0±0.2 (4)	P<0.005
6	+	—		13.3±0.4 (4)	
7	—	+	+	8.3±0.5 (3)	N.S.
8	—	+	+	9.1±0.5 (3)	

FSH was injected to prepuberal rats 2 hrs before the start of incubation. Puromycin was injected i.p. 15 min before and 60 min after the FSH-injection. The ovaries were incubated for 2 hrs in Krebs bicarbonate buffer containing 0.1 mM AIB-^{14}C and 5.5 mM glucose. In groups 5—8 puromycin was also added to the incubation medium (0.5 mg/ml)

Ratio of cpm/ml intracellular water cpm/ml medium. Mean±S.E. Number of observations in parenthesis.

30 min before the FSH injection, and puromycin (5 mg) was injected i.p. 15 min before the FSH injection. One group of rats was injected with the aminonucleoside of puromycin (5 mg i.p.) instead of puromycin. The ovaries were incubated in buffer containing 0.1 mM AIB-^{14}C and 5.5 mM glucose, and AIB-^{14}C distribution ratio and lactic acid production were measured after 2 hrs of incubation. The results are shown in Fig. 6 and it is clearly seen that also under this condition puromycin blocked the effect of FSH on the AIB-uptake. The aminonucleoside of puromycin,

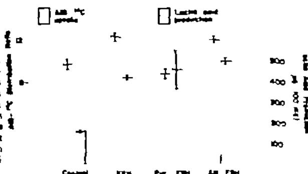


Fig. 6. Accumulation of AIB and production of lactic acid by ovaries from prepuberal rats. All rats were anesthetized with nembutal i.p. (4 mg/100 g b.w.) 1 hr before the start of incubation. FSH was injected (500 μg/100 g b.w.) 30 min before the incubation. Puromycin (Pur) or the aminonucleoside of puromycin (PAN) was injected i.p. (5 mg) 15 min before the FSH injection. Each column is the mean of 3—4 observations and the standard errors are indicated on the top of each column. The effect of FSH on AIB-uptake is significant in groups 2 and 4 (P<0.05) but there is no effect in group 3. The effect of FSH on lactic acid production is significant in groups 3 and 4 (P<0.02).

however did not have this effect. It can also be seen that the ovaries from the FSH-injected rats produced more lactic acid under the incubation period than simultaneously incubated ovaries from control rats, and that this effect of the hormone was not blocked by puromycin.

Discussion

The use of the amino acid analogue AIB as a model for amino acid transport across the cell membrane has been criticized since the kinetics of AIB accumulation in many cells differ from those of other amino acids. For example Segal *et al.* (1966) recently reported that thyroid gland slices continued to increase the intracellular concentration of AIB nearly linearly during 4 hrs of incubation, while normal amino acids as well as the non-utilizable amino acid cycloleucine reached steady state levels in the tissue after 30 min.

It can be seen from the results of the present study that the cells of the isolated rat ovary also continued to increase the intracellular concentration of AIB nearly linearly for very long periods (at least up to 4 hrs) and that the kinetics of the uptake of this amino acid analogue also in these cells seem to differ from those of other amino acids. A more rapid uptake and a steady-state distribution after 30–60 min was found with the amino acid analogue cycloleucine. The finding that the ovaries from FSH injected rats showed both an increased rate of uptake of AIB and a higher level in the steady-state distribution of cycloleucine in our opinion supports the conclusion that this effect of FSH has a physiological significance.

When FSH was injected 2 hrs prior to incubation a clear dose response was found (see Fig. 4). This fact is another indication in favour of the interpretation that the effect of FSH on amino acid transport has a physiological significance and it also suggests the possibility to use this effect as a bioassay method for FSH. The most common[illegible] used, and probably the most specific method used as a bioassay for FSH at present is the ovarian augmentation test originally described by Steelman and Pohley (1953). This test refers to the weight increase in the ovary as a result of hormonal injection for several days. A bioassay method with only one injection of the hormone 2 hrs before the [illegible] would therefore be a great improvement. Further [illegible] is necessary however to investigate more in detail both the specificity of the effect of FSH on amino acid transport and the optimal experimental conditions for a bioassay method.

Ahrén and K[illegible] (1963) mentioned that FSH did not stimulate the AIB-uptake in the rat ovary when the hormone was added directly to the incubation medium, and this lack of [illegible] effect has since been confirmed in subsequent studies (Ahrén and Rubinstein 1965; Noble and Kostyo 1965) as well as in the present experiments. Various possibilities to explain this lack of *in vitro* effect have been suggested (Ahrén and Kostyo 1963). FSH may not be able to reach its target cells in the whole isolated ovary when added *in vitro*, the hormone may need to be modified in some way by the organism before it can stimulate its target cells, diffi-

nonal humoral factors may be essential in the action of the hormone on the ovary. This lack of *in vitro* effect now seems even more interesting in relation to the finding of the present study in which an i.v. injection of FSH for as short a time as 5—7 min before the start of incubation had a stimulatory effect on the AIB-uptake under the subsequent incubation period.

The possibility that FSH may not be able to reach its target cells in the isolated whole ovary cannot be excluded. The fact that LH, a polypeptide hormone of about the same size as FSH, and also FSH itself in higher concentrations, can stimulate glucose uptake and lactic acid production when added *in vitro* to isolated rat ovaries (Ahrén and Kostyo 1963, Armstrong, Kilpatrick and Greep 1963, Hamberger and Ahrén 1967) illustrates, however, that the gonadotrophins can have certain stimulatory effects *in vitro*.

The possibilities that FSH might need to be modified in the organism before it can stimulate the ovarian cells or that additional humoral factor(s) must be present, have to some extent been explored by Noble and Kostyo (1965). They found that incubation of prepubertal ovaries in plasma from FSH-injected rats did not stimulate the rate of AIB-uptake. They also added other hormones (LH, growth hormone, oestrogens) directly to the incubation medium together with FSH, but none of their hormonal combinations had any effect on the AIB-uptake. It was therefore impossible for these authors to present an explanation for the lack of *in vitro* effect of FSH on amino acid transport.

In the present study it was found that pretreatment of the rats with puromycin blocked the effect of FSH on amino acid transport. It is well known that puromycin inhibits the protein synthesis in mammalian cells (*e.g.* Yarmolinsky and de la Haba 1959) and probably acts by combining with polypeptides in the course of protein synthesis thereby causing release of incomplete peptide chains (Nathans 1964). It is, however, also evident that puromycin in some systems (*e.g.* liver, fat tissue and isolated rat diaphragm) has effects which are not directly related to its influence upon protein synthesis (*e.g.* Hofert *et al.* 1962, Korner and Raben 1964, Sovik 1966). These unspecific effects of puromycin can also be elicited with certain puromycin analogues, *e.g.* the aminonucleoside of puromycin (PAN), which have no or very weak inhibitory effects on protein synthesis. No inhibitory influence of PAN on the effect of FSH was seen in the present experiments, and it seems therefore most likely that the inhibitory effect of puromycin in our experiments is a consequence of its effect on protein synthesis. The most probable interpretation of the experimental data available at present seems thus to be that there is at least one phase in the sequence of events leading to a stimulation of amino acid uptake by FSH in the rat ovary which needs an active synthesis of protein. This phase might occur in the ovary itself, *e.g.* in the process of binding of the hormone to the cell receptor sites but this might as well be located elsewhere in the organism. Further experiments are necessary both to prove whether our interpretation of the results with puromycin is correct and to demonstrate where in the body the inhibitory effect of puromycin is located.

The fact that LH stimulates glucose uptake and lactic acid production by isolated prepuberal rat ovaries both when the hormone is injected to the animals prior to incubation and when it is added directly to the incubation medium has only briefly been mentioned above. This effect has been analysed in detail in another study (Hamberger and Ahrén 196[illegible]). In that study it was found that FSH also stimulated glucose uptake and lactic acid production in the isolated ovary when the hormone was added to the medium in high concentrations (10—1000 μg/ml medium of NIH FSH S2). The dose response relationship could not be explained on the basis of a recognized contamination of the FSH preparation by LH. It is seen from the results of the present experiments that injection of FSH (500 μg/100 g b.w.) to the prepuberal rats, both 2 hrs and 5—7 min before the start of incubation, stimulated the lactic acid production under the subsequent incubation period. Further studies are necessary to clarify if this *in vivo* effect of FSH can be explained on the basis of an LH contamination or if it is an effect of the FSH molecule itself. It is in any case of interest that the effect of the injected FSH preparation could not be blocked by puromycin in contrast to the effect on AIB transport. Further studies with puromycin seem therefore to offer a suitable experimental procedure to differentiate and analyse the significance of various effects of the gonadotrophins on the ovarian metabolism.

We wish to thank the Endocrinology Study Section of NIH for the generous gift of ovine FSH (NIH FSH-S[illegible]) and the F[illegible] Ltd Malmö for the supply of most of the [illegible] for the [illegible] diet.
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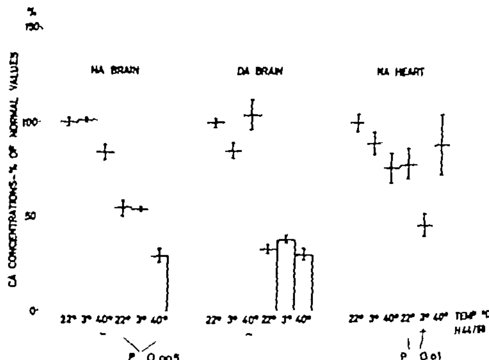


Fig. 2 The effect of environmental temperature on the level of NA in rat brain and heart and of DA in rat brain after inhibition of the CA synthesis by H 44/68 (250 mg/kg i.p., 3 hrs before killing. The data are expressed as percentage of normal values ± s.e.m. of 3–4 separate expts. The rats were kept at the different temperatures for 3 hrs. For body temperature of the rats, see text.

TABLE I. NA concentrations in heart and NA, DA and 5 HT concentrations in rat brain 5 hrs after H 22/54 500 mg/kg i.p. Administration to rats kept at + 5° C or + 23° C. (Per cent of normal values ± s.e.m.)

	Heart	Brain		
	NA	NA	DA	5 HT
Untreated	100.0 ± 5.0	100.0 ± 2.0	100.0 ± 2.5	100.0 ± 3.9
5°	61.0 ± 9.5	48.7 ± 3.1	47.9 ± 3.0	58.6 ± 4.3
23°	99.8 ± 5.1	43.0 ± 1.3	42.6 ± 3.5	58.3 ± 2.0
Significance of difference between values + 5° C and + 23° C	p 0.01	p > 0.05	p 0.05	p < 0.01

temperature after H 22/54 (Table I). Histochemically mainly the following areas were studied: the spinal cord, the medulla oblongata (e.g. nuc. hypoglossi, nuc. motorius dorsalis n. vagi), the pons (e.g. nuc. motorius n. trigemini, nuc. n. facialis) and the hypothalamus (e.g. the nuc. suprachiasmaticus) where the number and

intensity of fluorescence of the 5-HT nerve terminals are easily evaluated) There was a retardation of depletion of amine in the 5-HT nerve terminals of rats kept at + 3 C when compared to those of rats kept at room temperature. This retardation appeared to be most marked in the hypothalamus and the lower brain stem of the sha ed rats (see also Fig 1) In these animals large numbers of 5-HT nerve terminals still emitted a moderat yellow fluorescence in the parts of the brain studied. In the non-shaved rats effects were mainly observed in the 5-HT nerve terminals of the hypothalamus, *e.g* in the nuc. suprachiasmaticus. It should be pointed out that the shaved rats, which exhibited the best retardation of depletion usually had a severe hypothermia (+ (23 —24) C, rectal temperature) The non-sha ed rats, which showed much less retardation of depletion of 5-HT had a considerably higher rectal temperature (about + 30° C) The central NA and DA nerve terminals, on the other hand, did not show any clearcut changes in their rate of amine depletion after H 22/54 under the influence of a cold environment and not even in a state of severe hypothermia. The peripheral drenergic neurones innervating the heart, however showed a marked acceleration of NA depletion at + 3 C (Fig 2) (Corrodi and Malmfors 1966)

Further in a warm environment (+ 40° C) changes occurred in the rate of de pletion of 5-HT after H 22,54 (Fig 1) In this situation, there was an increased rate of depletion of 5-HT and the rats exhibited hyperthermia (+ (40—41) C) At + 30° C no certain changes were observed in the rate of depletion of 5-HT Histochemically there was a definit acceleration in the degree of depletion in the 5-HT nerv terminals at + 40° C. Only nerve terminals showing a pale yellow fluorescence remained and large numbers of nerve terminals emitting a yellow fluorescence had completely disappeared in the areas studied. These effects seemed to be less pronounced in the spinal cord The rats exhibiting the most severe hyper thermia showed the greatest increase in rate of depletion of 5-HT as compared to that of rats kept at + 23 C Also the NA, but not the DA, nerve terminals of the brains of rats kept at + 40° C showed clea to marked (severe hyperthermia) increases n the depletion rate after H 22/54 Thus, only NA nerve terminals which showed a pale to medium green fluorescence remained in a large number of areas in the brain (g hypothalamus medulla oblongata) Usually numerous NA terminals with medium to bright green fluorescence were still observed in these parts after H 22 54 if the rats had been kept at normal room temperature

H 44 68-experiments The biochemical results are summarized in Fig 2. As seen, these show that cold (+ 3) did not seem to influence the degree of depletion of NA and DA in the brain but only the depletion of NA in the heart (see also Corrodi and Malmfors 1966) Histochemically the NA and DA nerve terminals in th brain showed about the same degree of depletion as in rats kept t + 23 C The rectal temperature of the rats kept t + 3 C was around + 36 C

Heat, on the other hand caused marked increase in the degree of depletion of NA but not of DA in the brain after treatment with H 44 68. The NA content in

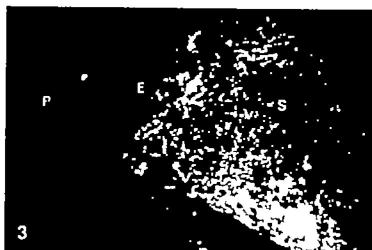




Fig 3 Nuc motorius dorsalis g and uc tractus solitarii of rat after treatment ith H 44/68 (250 mg kg p 3 hrs before killing) t + 23 C A collection of weakly to strongly green-fluorescen NA nerv t rminals are observed. E = Ependyma P = Plexus chorioideus S = Nuc act solitarii V = Nuc motorius dorsalis gt. × 200

Fig 4 Nuc motorius dorsalis gi and nuc tractus sol tarii of rat after treatment ith H 44/68 50 mg/kg p 3 hrs before killing) t + 40 C. Only few green-fluorescent nerve terminals still observed due increased depletion of amine from the NA nerv terminals his emper For bbreviations see text to F g 3 × 200

the heart howev d d not how any certain change in rate of depletion. The histochemistry also evealed marked increases in the rate f depletion of various NA terminal systems g the limbic forebrain structures, the hypothalamus (Fig. 5 and 6) the pons and the medulla oblongata (Fig. 3 and 4) The NA nerve terminals innervati g th nuc supraopticus and the nuc. para entricularis were clearly less affected than g those in the lateral hypothalamus and in the periventricular system. The effects in the spinal cord seemed to be somewhat less pronounced The rectal temperature of these rats was around + 40° C.

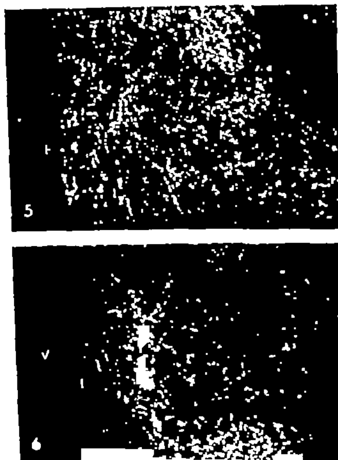


Fig. 5. Nuc. dorsomedialis hypothalami of ra after treatment with H 44 68 (250 mg/kg, i.p., 3 hrs before killing) t + 23 C. A collection of weakly to strongly green-fluorescent NA nerv terminals re observed. E = Ependyma. V = Third entricle. × 200.

Fig. 6. Nuc. dorsomedialis hypothalami of rat after treatment w h H 44 68 (see text to fig. 5) at + 40 C. A considerably lower number of NA nerv terminals is observed than in Fig. 5 due to increased amine depletion. For abbreviations, see Fig. 5. × 200

D scussion

Strong support has been obtained for the iew that the central NA and 5-HT terminal systems hange their stat of ctivity in large number f areas n he brain g in the hypothalamus if exposed to hanges in environmental temperat re.

It was found that the central NA neurones markedly increased thei rate of depletion of amine fter H 44 68 and H 22 54 if exposed to heat – 40° (b t no t cold + 3 C). Thus, the activity f the central NA neurone is p obabl re sed during heat but not during cold exposure. The scending NA neu on es f m the rhombencephalon (Andén *et al.* 1965) appeared to show th bes degree f activa tion, since the NA nerv terminals of the pinal ord did not seem t be as clearly

affected as those in the brain stem. None of the central DA nerve terminal systems, however were either influenced by heat or by cold. Furthermore the peripheral NA neurones innervating the heart in contrast to the central NA neurones, were not activated by heat From this and a previous study (Corrodi and Malmfors 1966) evidence ha been presented that these peripheral NA neurones innervating the heart are instead activated by cold Thus the central and peripheral NA neurones react differently to changes in the temperature of the external environment, indicating that they may have a different thermosensitivity The 5-HT neurones, on the other hand, showed a retarded rate of amine depletion if the rat was exposed to cold and an increased rate of depletion if it was exposed to heat indicating a decreased and increased activity in these neurones during cold and heat exposure respectively These effects seemed to be most marked in the ascending 5-HT neurones (Andén *et al* 1965) since the changes in the 5-HT nerve terminals in the spinal cord appeared to be less pronounced

The effects observed in the 5-HT neurones and the central NA neurones after treatment with H 22 54 and H 44 68 in the present experiments must be interpreted with caution since *inter alia* thermoregulation was out of order In fact, the possibility cannot be excluded that the changes observed in the degree of depletion of the 5-HT and NA nerve terminals are due to general metabolic changes and not to activity changes since severe hypothermia and hyperthermia respectively was present. However the facts that th central DA and NA nerve terminals did not show any retarded depletion during hypothermia, that the NA terminals of the heart showed an increased rat of depletion in spite of severe hypothermia and that the DA and peripheral NA nerve terminals showed no increased rat of depletion during hyperthermia is contrary to this possibility Thus, it is probable that th changes observed represent acti ity changes

It can be rgued that the activity changes observed are due to stress and ha e nothing to do w th thermo-regulatory responses. This argument may be valid for the acti it changes n the central NA but not in the 5-HT neu rones, since preliminary dat ndicat that immobilization stress (Corrodi Fuxe and Hökfelt unpublished i ncr ses the acti ity in the central NA but not in the 5-HT neurones. Furthermor th cent al NA neurones probabl also ha an increased activity during exercise (C d n *t al* 1966) The fact that the central NA neurones are not activated by ld xposure however favours the iew that both the central NA neurones and the 5 HT neurones are concerned with temperatu regulation.

According to Feldl rg and My r (1964 1965) an rea exist in the anterior hypothalamus, whicl sensiti e to 5-HT NA and A (see introduction) It was proposed that th rmo gulatio n is maintained by the relative amounts of 5-HT and NA released from the NA and 5-HT terminals in this region to act on this area in the anterior hypothala nus In the cat and the dog ntraventricularly ad ministered 5-HT will in e se body temperature whereas NA will decrease it. Whether this applies in the case of the rat is not known. There is little doubt, however that intraventricularly dministered NA and 5-HT are taken up partly into

NA and 5-HT nerve terminals respectively in the part of the hypothalamus lying close to the third ventricle (Fuxe and Ungerstedt 1966). In the present experiments no change in activity was revealed in the central NA neurones during cold exposure, indicating that these respond only to an increase in environmental temperature whereas the 5-HT neurones seem to respond to both decreases and increases in environmental temperature. The changes observed in release of NA and 5-HT are probably of mainly functional importance in the hypothalamus where the thermosensitive centre is localized. Nevertheless, the activity changes observed in the other areas may also be of importance, since these may modify the afferent input (e.g. that from the reticular activating system) to the thermosensiti e centre.

According to the present data hypothermia may be associated with decreased activ ty in the 5-HT but not in the central NA neurones, and hyperthermia with increased activity in the central NA and 5-HT neurones. If we assume that as in the rabbit and the goat (Cooper Cranston and Honour 1965 Andersson, Jobin and Olsson 1966) 5-HT is able to decrease body temperature in the rat when administered intra entricularly it seems likely that the increased activity observed in the 5-HT neurones at exposure to heat represents a response to counteract hyper thermia. Similarly the decreased acti ity observed in the 5-HT neurones at exposure to cold may represent a response to counteract hypothermia At any rate these events do occur *inter alia* at thermoregulatory synaptic s tes in the anterior hypothalamus and are probably of importance for proper functioning of the thermosensitive hypothalamic centre. Whether the effects observed by Feldberg, Myers and others with intraventricularly administered NA nd 5-HT are specific or not in view of the high amine concentrations used remains to be seen, but the present results do in dic te that the central NA and 5-HT neurones participate in temperature regu lation Of course, non-monoamine-containing neurones may also be invol ed.

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The Relationship between Active Constriction and Passive Recoil of the Veins at Various Distending Pressures

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Abstract

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The vasoconstrictor fibre influence on veins, exposed to varying distending pressures, was analysed with respect to the relative contribution of active venoconstriction and passive venous recoil, respectively, to total venous blood mobilization. The results indicate that active venoconstriction can produce only a minor reduction of venous capacity when venous transmural pressure is low but a considerable diminution of the capacity at higher venous pressures, provided the pressure is not so high as to overstretch the muscle elements. — The passive emptying of blood on vasoconstrictor fibre stimulation is very pronounced in the low pressure range but becomes small at higher pressures, in accordance with the pressure-volume characteristics of the veins, demonstrating an apparently very high distensibility at low pressures. This high distensibility is mainly due to changes of vessel geometry associated with the collapse and does not reflect true wall distensibility. — Consequently, when venous pressure is low, venous function is primarily governed by passive factors, while active shortening of venous smooth muscles will play a dominant role first when the vessels are kept well distended.

The implications of these findings for venous control is discussed, with special respect to man, where venous pressure varies greatly with body position. Some methods earlier used for assessing venous tone changes are critically analysed.

The venous side of the circulation does not only serve as a passive return route for the blood to the heart but can also be considered as an adjustable depot, containing 70—75 per cent of the total blood volume (e.g. Green 1950, Wiedeman 1963). Alterations of the blood content in this large capacitance section of the cardiovascular system will evidently most strongly affect venous return and the "filling" of the heart, hence cardiac output and overall circulatory dynamics. The various mechanisms controlling venous blood content must therefore constitute important tools to maintain cardiovascular homeostasis (cf. Folkow and Mellander 1964).

It is well known that venoconstriction, with consequent blood mobilization, occurs in most vascular beds upon vasoconstrictor fibre activation, whether this is induced by direct electrical stimulation (Mellander 1960) or in connection with various cardiovascular reflexes (e.g. Öberg 1964). Such active adjustments of the veins

are however inevitably accompanied by a more or less pronounced passive blood mobilization whenever the precapillary neurogenic constriction is pronounced enough to decrease the transmural pressure within the postcapillary section.

The magnitude of such "passive" adjustments must, however, vary considerably with the prevailing venous transmural pressure since the distensibility of the veins varies greatly with the pressure level. Thus, in the low pressure range, even a minor pressure fall is followed by a marked volume reduction, as indicated by the well known convexity of the venous pressure volume curve towards the volume axis (for references, see Alexander 1963). From this curve one no doubt gets the impression that venous "distensibility" is very high in the low pressure range. However it is likely that volume alterations here mainly reflect a change in venous transectional geometry from a circular to an elliptic shape, i.e. a tendency of venous collapse rather than a passive-elastic recoil of the venous wall (e.g. Burton 1965).

As a contrast, within the higher pressure range, where the veins attain a circular transectional area, even pronounced shifts in transmural pressure are followed by only small changes in volume. However, these volume changes can be expected to reflect the true wall distensibility.

One might further expect that even pronounced contractions of the venous smooth muscles, following a vasoconstrictor fibre activation, can produce only small blood mobilizations from the veins when the transmural pressure is initially so low that the veins are collapsed and therefore almost void of blood. Accordingly it may be predicted that in the low range of venous transmural pressure alterations in venous blood content is primarily governed by "passive" factors.

At higher pressure levels, on the other hand, when the veins are well distended and display a circular transectional configuration, volume changes may be mainly ascribed to active shifts in smooth muscle length, while passive adjustments will now be of minor importance due to the relatively low venous distensibility. The expected transformation of the functional characteristics of the veins with the transmural pressure must have important consequences for the regulation of venous ret rn especially in man, where the major part of the venous reservoir is placed at heart level in the supine position but well below the heart in the erect position.

Further, the mentioned characteristics of venous function must imply that quantitative analyses of the nervous control of the veins, when recorded in terms of shifts in blood content and or pressure, are met with considerable difficulties. Such studies are however of great importance since the true nature of reflex patterns governing circulatory homeostasis can be fully understood only when the extent of activation of the various cardiovascular effectors is quantitatively estimated. As mentioned above, vasomotor fibre activations imply not only "active" venoconstriction but also a superimposed passive blood mobilization whenever venous transmural pressure becomes lowered as a result of precapillary vasoconstriction. This is seen e.g. in the skeletal muscle vascular bed, where a reflex increase in the pre- to postcapillary resistance ratio leading to a fall in capillary and hence in venous pressure occurs when the arterial baroreceptors are unloaded and/or the

chemoreceptors activated (Öberg 1964) For an accurate quantitative evaluation of the active venoconstriction in these reflex patterns one must thus know the exact contribution of passive emptying This is, as mentioned related not only to the absolute fall in venous transmural pressure but also to the prevailing level of this pressure.

Some of the mentioned problems have earlier been analysed in an interesting study by Thron and Scheppokat (1958) on hand veins in man. However in studies of this type on man it seems impossible to rule out all sources of error and it is difficult to ensure that equally strong reflex activation of the vasoconstrictor fibres is repeatedly induced. In the present study several different techniques have been utilized in an attempt to estimate quantitatively the relationship between "active" and "passive" venous reactions at various transmural pressures when the venous smooth muscles are exposed to exactly defined neurogenic stimuli.

Methods

Experiments were performed on 35 cats, anesthetized with chloralose, 30—50 mg/kg body weight

I Experiments on isolated veins

A series of studies concerning the pressure-volume characteristics of the veins were made on isolated segments from the inferior caval vein, the femoral, the superior mesenteric and the jugular vein. One end of the horizontally placed vessel segment was cannulated and connected to a Statham pressure transducer for pressure recordings on a Grass polygraph The other end was connected to an infusion pump, delivering a constant volume of fluid (blood or dextrane) per unit time The time axis on the recording will thus be synonymous to volume axis

In these experiments attempts were made to correlate the pressure-volume characteristics of the veins also with alterations in their transectional "diameter" This was done by measuring the distance between the two most remote points of the same circumference of the initially collapsed vein, when observed from above, and how this "diameter" changed when the transmural pressure was slowly increased above zero. A reduction of this diameter upon pressure and volume increases should then imply a change of the transectional geometry from an elliptic to a more circular form — Changes of the vessel diameter were assessed by direct inspection from above through a low magnifying dissection microscope, equipped with an occular with an engraved scale For each observed 0 2 mm change of vessel diameter a mark was made on the polygraph paper with the pressure recording.

After the experiment the length and circumference of the venous segment was carefully measured These values were used for a deduction of its volume, had it attained a perfect circular shape at zero transmural pressure This "zero pressure-volume" was used as a reference point to evaluate approximately at what pressure level the cross section of the vein attained an oval shape and for a rough determination of the genuine wall distensibility at very low transmural pressures

II Experiments on intact vascular beds

A *Hindquarters preparation.* The reactions of the veins ... were in most cases studied in the hindquarters, by means of a plethysmographic technique, earlier described in detail (Mellander 1960) Briefly the abdomen was opened, the intestine removed and the inferior caval vein and the aorta were dissected free All side branches of these vessels from the bifurcations to the renal vessels were ligated and divided Mass ligatures were placed around the muscles of the abdomen and the back, and the spinal channel was plugged to obstruct its veins The hindquarters were thus separated from the rest of the animal except for the vertebral column, the sympathetic trunks, the aorta and the inferior caval vein the latter being the sole venous outflow channel from the preparation

The hindquarters were placed in a plethysmograph filled with water kept at 37 C A circular skin flap was freed from the underlying tissue at the abdomen and the back and

t a fi ed by ros ing flange nd screws round the opening of the plethysmograph, so as t mak the plethysmograph water tight The plethysmograph wa connected to a piston recorde which registered t sue volume ha ges on smoked paper Rapid phasic changes of l me c n with this technique be scribed t shift in enou blood content, while slower ontinuou olume ls tion be ref rred t t ncapillary fluid movements (Mellander 1960)

The sympathetic trunk to the hindqua ters wer dissected free cut centrally and placed n b pola ele le trodes for peripheral stimulation. Stimuli ere deli red from Grass tumul tor A terial blood pressure was recorded from the inf rior mesenteric artery By adjustment of screw lamp placed on the aorta proximal to the site for the pressure re rding the inflow pr ssu t the hindquart ould be adjusted t any desired level — Blood flow wa me sured by l ting the cav l ein and diverting the total enous efflux through d op- ecorde ope ting n ordinat writer or through modified Gaddum re rder The blood w ret rned to the a im l th ough the ent l end of the caval ein The height t wh h the flow-recorder w placed bov the w ter lev l in the plethysmograph epresented the enou outflow pressure which thu could be aried or kept constant at will

In some expe iment the hindqua ters were perf sed by onstant flow delivered b finger pump (H rv d Mod l 500- 1900) The abdominal aorta was then divided and the blood was pa sed ia plastic tubing through the pump from th proximal to the distal end of the aorta

I some expe ime the enous reaction were studied in the intestine which was prepared according t th technique described by Folkow Lundgren nd Wallentin (1963) A suitable length of th small tes ine w isolated nd the mesenteric root, carrying the essels and ne es the prepa tion w ref lly freed from adjacent struct res so that long and flexible pedicle was ta ned The mesent ric ein was annulated and the outflow was pa sed th ough drop- orde for flow measurement The blood was returned la the right j g la ein A small ein in the mesentery w nnul ted and connected t Statham pressu sducer ecording enous pressure on Grass polygraph

Th testine w placed in plethysmogr ph with the mesenteric pedicle and the pressure ubing from the sm ll n aref lly positioned in w ll fit ing opening in the plethysmograph The open g w filled w th w ter epella t grease to sure complete te -tightness — The plethysmog ph w filled with Tyrode solution kept t 37 C and connected to piston ecorde for olume regist ion The plexu of sor nstrictor fibres running along the mese ter rt ry w ef lly prepared free nd pl ed on lectrodes for stimulation Volt g nd pulse d t n w so adj sted t produ maximal scular responses for the f quency lsed

B l ee p m ment I one series of experiment the extent of acti e constriction w ssessed f om the rise in pressu within the isol ed int stinal seeds bed where it la ion w temporaril reated by placin lamps on both the rtery and the ein Prior the lamping the no outflow pressure had been set at diff rent levels so that he isolat d scula bed ould either be more or les ollapsed or well filled with blood ffected by the qu libr ed pressure lev l Setting ou from hese rying pressure levels he socons fibr we imulated in standardized wa nd the resulting pressure ch w re rded P ssu w monitored from small side branch of the mesenteric vein by mean of St h m pressure t nsducer

Results

I Expe m nt n solat d enous gm nt

Fig 1 how the rec rd ngs from a typical experiment where the pressure-volume chara teristics f n isolated inferior caval vein was correlated to changes in the transectional geomet y of the vessel. The curve show the ell known convexity towards the olume

In the low p es ra ge between 0 to about 8—10 mm Hg even large volume increments produce only small pressure increases, *i.e.* the distensibility is high However once pressure lev ls above 8—10 mm Hg are reached, the pressure increase for a given olume increment becomes more pronounced, *i.e.* the distensibility is here relati ly low The simultaneous registration of changes in cross

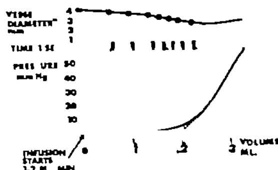


FIG. 1 Changes of pressure in and "diameter" of an isolated segment from the inferior caval vein with increasing fluid content. Segment length and circumference 4 and 0.8 cm, respectively. Zero pressure volume provided perfect circular transectional configuration 0.2 ml

diameter (see Methods) shows that there is a continuous *reduction* of this dimension, more slow at the beginning but with augmented speed when the volume increases, up to a point where the diameter tends to stabilize and then starts to increase somewhat. At this point, where the diameter decrease changes into a diameter increase, the vein should have attained a circular transection configuration. The volume of fluid added up to this point slightly exceeded the zero pressure volume of the venous segment as deduced from the measurements at the end of the experiment (see Methods) — It can be seen that the point where the vein attained circular transectional configuration, corresponds roughly with the bend in its pressure-volume curve where the pressure starts to rise more markedly at 8—10 mm Hg

This finding will be briefly commented already in this connection, since it has some direct bearing on the results described below It suggests that pressure dependent volume changes of the veins in the low pressure range is accomplished mainly by geometry changes, and that a minimal transmural pressure actually as high as 8—10 mm Hg in this experiment, is necessary to keep the veins in a fully circular shape This minimal transmural pressure value, where thus the veins become circular and the venous pressure starts to rise more significantly varied somewhat in the different preparations but were throughout *in the range of* 6—11 mm Hg It might therefore be permitted to draw the conclusion that, in the cat, transmural pressure below some 6 mm Hg in the larger veins implies a certain degree of collapse Therefore the part of the pressure-volume curve, corresponding to the pressure range below 6—8 mm Hg does not reflect the true wall distensibility but mainly changes in luminal geometry True wall distensibility at these low pressure levels is better illustrated if the curve is extrapolated back to the zero pressure volume the volume that would have been attained by the vein at zero pressure had the transectional area been kept perfectly circular throughout dotted line in Fig 1

II Experiments on intact vascular beds

A Venous volume measurements Fig 2 shows an experiment where blood flow and volume changes in a hindquarter preparation were followed when the regional vasoconstrictor fibres were intermittently stimulated with identical stimulation characteristics (4 imp/sec 3 V 3 msec) but at varying levels of mean venous transmural pressure produced by alterations of venous outflow pressure It can be seen that the initial rapid volume decrease following stimulation, which reflects mobilization of blood from the hindquarters, and then predominantly from the veins, *dec eases* somewhat when the venous transmural pressure *increases*. Thus, while the blood mobilization amounts to roughly 4.5 ml when venous outflow pressure is 4.5 mm Hg it is in this experiment only 3 ml when venous outflow pressure is set at 16 mm Hg and less than 2 ml at a venous outflow pressure of 22 mm Hg

The responses of the resi tance vessels, as reflected by the shifts in flow resistance (PRU) are if anything enhanced when the venous pressure is raised. The diminution of the volume responses at the high levels of venous transmural pressure can therefore not be attributed to a less ffective vasoconstrictor fibre stimulation, nor to a less pronounced volume reduction of the precapillary vascular sections. Consequently it must be related to a less effecti e emptying of the venous compartment.

It should be realized that even if the outflow pressure is kept constant during the stimulation period the *m an* venous transmural pressure being approximately half

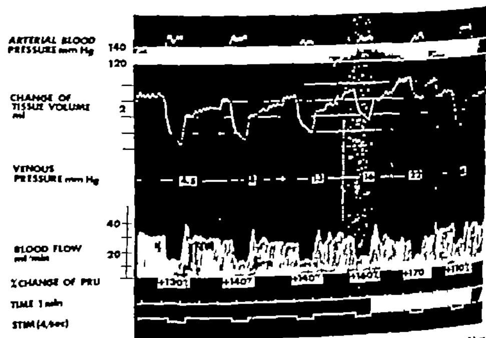


Fig. 2 Cat 2.6 kg Hindquarte preparation Effects of standardized vasoconstrictor fibre stimulations on blood flow nd issue volume t varying levels of enous transmural pressure

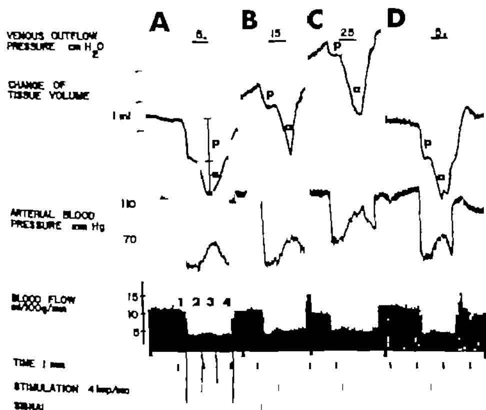


Fig. 3 Cat 3.1 kg Hindquarter preparation.
Panel A Passive (p) and activ " ( ) tissue olume changes produced by partial occlusion of the aorta (line 1) and asoconstrictor fibre stimulation while keeping blood flow constant (line), respectively t low venous distending pressure
I *Panels B C and D* the same procedure is repeated t moderate high and again low venous transmural pressures.

the sum of the capillary and the venous outflow pressures, must inevitably fall when precapillary vessels constrict, since the capillary pressure then falls. This capillary pressure fall is revealed in the volume record in Fig ? by a net absorption of tissue fluid during the stimulation periods, as indicated by the slower phase of tissue volume reduction follow ng the fast, initial enous response. Therefore the emptying of blood from the veins, as shown in Fig 2, is due to both an *ct ve* venoconstriction and to a *passiv* elastic recoil of the veins. Since the precapillary constriction, if anything, seemed to be even more powerful at the higher range of venous outflow pressures, the reduction of mean venous pressure secondary to the stimulation, ought to be correspondingly more pronounced t these higher pressures. Despite this the total blood mobilization from the eins is then lightly smaller It can therefore be concluded that the reduced venous emptying must be due to less efficient active venoconstriction and or to a reduced passiv recoil of the eins for a given pressure reduction, i.e. to reduced venous distensibility

To analyse in approximate terms the quantitative relationship between these "active" and "passive" components, respectively which together constitute the total blood mobilization from the venous compartment, experiments of the type shown in Fig 3 were performed. Changes in blood flow and tissue volume produced by standardized vasocon trictor fibre stimulations, were here studied in a hindquarter preparation at various levels of venous transmural pressure. Prior to each stimulation, the blood flow through the preparation wa reduced by a partial obstruction of the abdominal aorta (line 1 in panel A) to a level slightly less than that expected to be reached during the subsequent constrictor fiber stimulation. This addition of an artificial precapillary resistance produces a volume reduction (p at the volume registration in panel A) which must be ascribed solely to a passive mobilization of blood, mainly from the veins, as a result of the reduced venous transmural pressure. When a stable level of reduced blood flow and a reasonably stable volume had been attained after this manoeuvre a stimulation of the vasoconstrictor fibres was performed line By a graded opening of the aortic screw-clamp blood flow could be maintained constant during the stimulation period, despite the induced vasoconstriction. Thus, total flow resistance in the hindquarters is here kept constant by balancing the neurogenically induced resistance increase by a corresponding reduction of the artificial resistance applied via the screw-clamp However the volume recording now reveals a further blood emptying a consequence of the constrictor fibre stimulation (a at the volume registration This blood expulsion must be entirely due to a a tive venoconstriction since blood flow and venous outflow pressure are kept throughout constant This implies that, disregarding the possible influence of a slight increase of postcapillary flow resistance, the mean capillary and hence also the mean venous pressure must have remained essentially unchanged On cessation of the constrictor fibre timulation (line 3) the veins again dilat and when, lat r on, also the aortic screw-clamp is opened (line 4) the venous t a n ral pres ure build up and a passive filling of the capacitance section is added

The same serie of procedures are then repeated with venous outflow pressures set at 15 2 and again t 5 cm of water in panels B, C and D respectively As can be seen the pa on ponent of the venous blood mobilization (p at the volume recording dec es and the acti e one (a at the volume recording) increases with increa g ven pressures although their sum remains approximately the same at all lev l f venous transmural pressure studied

Fig 4 show the relationship between total, "acti and "passive blood mobilization a obt d f om an experiment of the type illustrated in Fig 3, performed on a hindq rt preparation. Essentially identical results were obtained in all experiment u th se ies including those experiments where a constant flow pump perfusion was sed It can be seen from this diagram that while the "total" blood mobilization if tling decreases slightly w th increasing transmural pressure within the venous comp rt ent as shown in Fig 2) this decrease is to be ascribed to the marked reduction f the pa s component of the blood mobilization, or

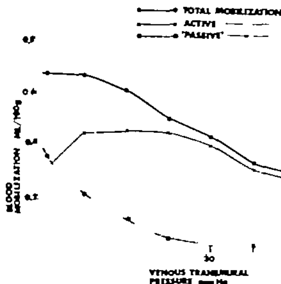


Fig 4 The influence of the prevailing enous pressure on the magnitude of *total ac tive* and *passive* blood mobilization, respectively following standardized vasoconstric tor fibre stimulations to th hindquarter preparation. — The data represent mean al- ues from 3 stimulations at each pressure level.

curring when venous transmural pressure rises. This marked reduction in the passive factor evidently coincides with the gradual elimination of the changes in transectional geometry of the veins associated with the collapse phenomenon, as outlined in section I On the other hand, the active emptying due to a shortening of venous smooth muscles, first *increase* when pressure rises, but decreases again when enous transmural pressure becomes higher than about 20 mm Hg

Essentially similar results were obtained in the intestinal preparations, utilizing the same experimental approach as utlined above However in the intestine the active enoconstrictions showed a tendency to reach their maximal response at a slightly lower level of transmural pressure than in the hindquarters.

B l enous p ss e m as rem ts In 5 experiments, an isolated intestinal ascular bed with a temporarily arrested circulation was utilized to analyse enous f nction at varying levels of distending pressure by recordings of the pressure changes evoked by standardized activations f the vasoconstrictor fibres Fig. 5 shows a recording from such an experiment Varying levels of tatic pressure in the closed ascula bed was ttained by varying the enous outflow pressure prior to the clamping of the vessels. It can be seen that the tent of the pressure rise, induced by aso-constrictor fibre timulation, is dependent upo the in tial enous pressure and th degree of distension of the eins Wl the in tial pressure is ery low so that the eins can be expected to be more r les llapsed, only ery small pressure rises re obtained upon sympathetic stim l Or he other hand, considerable pressure responses are obtained upon the sa e pa hetic stimulation when the initial pressure is set so high as to keep the s w ll distended. Qualitatively imila results were obtained in ll preparat ons in h serie

These data strongly suggest hat ignific t rise of enous pressure esul ng

To analyse in approximate terms, the quantitative relationship between these "active" and "passive" components, respectively which together constitute the total blood mobilization from the venous compartment, experiments of the type shown in Fig. 3 were performed. Changes in blood flow and tissue volume produced by standardized vasoconstrictor fibre stimulations were here studied in a hindquarter preparation at various levels of venous transmural pressure. Prior to each stimulation the blood flow through the preparation was reduced by a partial obstruction of the abdominal aorta (line 1 in panel A) to a level slightly less than that expected to be reached during the subsequent constrictor fibre stimulation. This addition of an artificial precapillary resistance produces a volume reduction (*p* at the volume registration in panel A) which must be ascribed solely to a *passive* mobilization of blood, mainly from the veins, as a result of the reduced venous transmural pressure. When a stable level of reduced blood flow and a reasonably stable volume had been attained after this manoeuvre, a stimulation of the vasoconstrictor fibres was performed (line 2). By a graded opening of the aortic screw-clamp blood flow could be maintained constant during the stimulation period despite the induced vasoconstriction. Thus total flow resistance in the hindquarters is here kept constant by balancing the neurogenically induced resistance increase by a corresponding reduction of the artificial resistance applied via the screw-clamp. However the volume recording now reveals a further blood emptying as a consequence of the constrictor fibre stimulation (*a* at the volume registration). This blood expulsion must be entirely due to an *active* venoconstriction since blood flow and venous outflow pressure were kept throughout constant. This implies that, disregarding the possible influence of a slight increase of postcapillary flow resistance, the mean capillary and hence also the mean venous pressure must have remained essentially unchanged. On cessation of the constrictor fibre stimulation (line 3) the veins again dilate and when later on also the aortic screw-clamp is opened (line 4) the venous transmural pressure builds up and a passive filling of the capacitance section is added.

The same series of procedures are then repeated with venous outflow pressures set at 15, 25 and, again, 5 cm of water in panels B, C and D, respectively. As can be seen, the passive component of the venous blood mobilization (*p* at the volume recording) decreases and the active one (*a* at the volume recording) increases with increasing venous pressures, although their sum remains approximately the same at all levels of venous transmural pressure studied.

Fig. 4 shows the relationship between total, active and passive blood mobilization as obtained from an experiment of the type illustrated in Fig. 3, performed on a hindquarter preparation. Essentially identical results were obtained in all experiments in this series including those experiments where a constant flow pump perfusion was utilized. It can be seen from this diagram that while the "total" blood mobilization, following decreases slightly with increasing transmural pressure within the venous compartment (as shown in Fig. 2), this decrease is to be ascribed to the marked reduction of the passive component of the blood mobilization, or

fibre discharge in such reflex adjustments. It is necessary to determine separately and with considerable exactness the extent of active venoconstriction. The present series of experiments is an attempt to evaluate quantitatively the contribution of *active* venoconstriction and *passive* venous recoil respectively to the total blood mobilization from a vascular bed exposed to different transmural pressures when the regional vasoconstrictor fibres are activated in a well-defined way.

The results of the present study indicate in agreement with those of Thron and Scheppokat (1958) that the magnitude of both the active and the passive component of venous emptying caused by vasoconstrictor fibre activation is markedly influenced by the prevailing venous transmural pressure. Thus, in the low range of venous transmural pressure the *passive* component of blood mobilization, secondary to a fall in venous pressure, is very pronounced, but becomes fairly insignificant at pressure levels above 5—10 mm Hg. This finding correlates well with the characteristic pressure-volume curve of the veins, which shows a very pronounced distensibility at low pressures and a considerably lower distensibility in the higher pressure range.

The present experiments on isolated venous segments show that the high distensibility in the low pressure range can be attributed mainly to the collapse of the veins i.e. to a change of transectional geometry rather than being an expression for the genuine wall distensibility. The steeper part of the pressure volume curve seems to correspond more closely to the true wall distensibility which in fact appears to be rather low. The detailed analysis of these pressure-volume curves further suggest that the true wall distensibility is fairly limited also in the low pressure range (see Fig. 1).

The *active* mobilization of blood, caused by standardized activations of the venous smooth muscles, was found to be very small when the venous transmural pressure was initially so low that the veins were collapsed or on the verge of collapse. Evidently a shortening of the smooth muscles in such more or less collapsed veins is unable to reduce effectively the volume of the vessel and even a strong active venoconstriction may in this situation, be completely obscured by the emptying caused by the passive luminal collapse for the veins. As hinted in this study a venoconstriction might then in fact even tend to slightly *increase* the vessel volume by stiffening the wall and in this way transform the collapsed vessel into a more rounded shape (cf. Wood and Eckstein 1958).

At venous transmural pressure level above 5—10 mm Hg, on the other hand the active emptying of the veins was found to increase both in absolute terms and especially when related to the passive emptying which decreased correspondingly when venous transmural pressure was raised. When the veins were exposed to pressures beyond approximately 20 mm Hg the magnitude of active blood expulsion again declined. The increase of the effect of the venoconstriction to standardized smooth muscle activation when pressure was raised from 5 to 20 mm Hg is probably related to the augmented resting length and tension of the smooth muscle, a characteristic feature of all muscle tissue including that in the vascular wall (S

en 1960 Sparks and Bohr 1962). The decrease of active venoconstriction at still higher pressure levels might be due to an "overstretching" of the muscle elements. It might seem surprising that an overstretching should be obtained already at such moderate pressure levels as 20 mm Hg. However, it should be pointed out that in cats, because of body position and size of the animal, the venous compartment is normally exposed to only moderate hydrostatic loads, rarely exceeding 10—15 mm Hg. The situation is most likely quite different in man where in the upright position the veins in dependent regions are normally exposed to a very high hydrostatic load. Accordingly, the veins in e.g. the foot of man have an especially well developed muscle layer (Kügelgen 1955) and it seems as if veins in general are structurally well adapted to the hydrostatic load that they are normally exposed to. It is therefore reasonable to assume that the maximal venoconstrictor response in e.g. the legs of man is obtained at definitely higher pressure levels than in the cat. As a matter of fact, Thron and Scheppokat (1958) found in their experiments on hand veins in man that the maximal active venoconstrictor responses were obtained in a pressure range between 30 and 40 mm Hg.

In most animals and also in man in the recumbent position, the greater part of the venous compartment is placed approximately at the heart level. The venous transmural pressure is therefore relatively low and especially more central parts of the venous compartment are probably more or less semicollapsed. Consequently passive adjustments of the venous reservoir will then play a rather dominant role while [illegible] active venoconstrictions cannot assert themselves significantly. This has the consequence that in this situation a fall in central pressure caused by e.g. hemorrhage [illegible] be very effectively compensated for by an active constriction of the venous compartment. It is even possible that whenever an active venoconstriction [illegible] rise in venous pressure this will be more or less completely offset by the simultaneous, pressure induced change of configuration of the [illegible] more rounded shape. This will tend to increase vessel [illegible] reduction of their circumference.

It [illegible] however that the average pressure distending the venous compartment [illegible] not identical with the central venous pressure [illegible] the mean of the capillary and central venous pressures. Normally [illegible] at the capillary end of the venous compartment is probably high [illegible] maintain the small veins distended and more or less rounded [illegible] where the pressure in more centrally located veins is so low as [illegible] collapse. However, in certain situations, as in circulatory shock [illegible] be expected to be quite low also in the small veins, due to a [illegible] of a reduced arterial pressure and an intense precapillary constriction. The [illegible] except a more generalized collapse of the total venous compartment [illegible] situation, and an active venoconstriction, however pronounced will then [illegible] very limited effect.

Studies of the present type inevitably give rise to speculations concerning the accuracy of the different methods commonly used for measuring changes of venous

tone. It is evident that a correct evaluation of the extent of acti e venoconstriction from changes in venous pressure or venous blood content can only be obtained when the veins are mod rately distended, and not n collapsed state as is the case in e.g. human forearm when placed above heart level or in isolated venous segments, almost void of blood (e.g. Alexander 1963). Fo the same reasons active venoconstriction may not be accurately revealed in supine man, when central enous pressure measurements are used for indicating shifts in venous tone (e.g. Gauer Henry and Sieker 1956).

The use of a decreased venous distensibility as demonstrated by an altered slope of the pressure volume curve as a criterion of venous mooth muscle construction, might also give erroneous results since according to the present study o ly the steeper part of the pressure-volume curve reflects the true wall distensibility. Further more since the apparent distensibility varies considerably w th the contained blood volume this volume must be exactly known to allow for correct evaluations and this has certa nly not always been the case.

A more detailed nalysis of such methodological problems is beyond the scope of the present tudy but will be discussed in a subsequent publication. However since the method for measuring tone changes of the veins by means f isotonic volume hifts, as used in the majority of the experiments in the present study (section II A) has been recently criticized in several respects (Browse Lorentz, Shepherd 1966) some comme its on this aspect seem justified. This mentioned method implies that quantitativ measurements of acti e venous constriction ha e been made under circum tances that allow for isotonic rather than isometric contractions. Such a type of enous contraction seems, in fact to conform better w th the situation in the ntact circulation, since contracting eins can pra tically always freely divert their content towards the heart other part of the cardiovascula system.

Isometric measurements, on the other hand, the utilization of pressure recordings from closed enous sections (g Browse t al 1966) seem to be in some respect less reliable. Th s, the enous smooth muscles re then forced to work under cond tions which are les phy iological and for whi l they are not primarily designed. Further the losed sy tem technique ines tably in olves circulatory stand till and risks for metaboli cumulation, which tends to counteract the neurogenically induced enocon trictions. There migl t lso occur a closure f enou al es, adjacent to the sit f h catheter so that measurements are made from n artificially limited, perhaps not representat part of the total enous compartment. Finally t is also l st possible to translat quantitative terms, the pressure increases, induced by given constrictor fibre t mul tion t the lume of blood that can be actu h obilized tow rd the entral eins d the heart by this same timulation.

The method of qua t tativ record ng f the isotonic enoconstrictions, used in most of the present experiment (sec ion II A) seems to ha con iderable advantages in ll bove-mentioned respect. It has, however been oked that, with the mentioned method it is not possible to diff rentiat betwee ct nd

"passive" blood mobilization from the venous compartment. As shown in the present study and as was already pointed out by Mellander (1960) such a differentiation is indeed possible and with considerable accuracy. It has also been argued that the present method does not clearly separate the blood mobilization occurring from the postcapillary venous section from that occurring from the precapillary vessels when the constrictor fibres are activated. This, too, is, however possible with a fairly high degree of accuracy by deductions from the data for the flow resistance increase, which to at least 80—90 per cent is known to reflect precapillary vessel constriction, and from data on the blood volume contained in the main resistance section of the precapillary vessels which appears to amount to less than 10 per cent of total regional blood content (Green 1950, Wiedeman 1963). If the flow resistance, being inversely proportional to the fourth power of the average internal radius, increases as much as 4 times, the blood content of the precapillary resistance vessels, being proportional to the square of the internal radius, would decrease to about half their initial volume corresponding to less than [illegible] per cent of the total blood content. This figure implies according to actual measurements as performed in this and similar studies (e.g. Mellander 1960) that only 10—15 per cent, at most, of total blood mobilization is derived from precapillary vessels and 85—90 per cent from the venous side if the small capillary blood content is considered to be largely constant. These deductions illustrate how it is, after all, possible to distinguish in fairly accurate terms also between precapillary and postcapillary capacitance responses also with the present method and that it is justified to relate blood volume shifts to alterations in venous capacity.

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Effects of Acetylcholine and Biogenic Amines on Pulmonary Smooth Muscle in the African Lungfish, Protopterus Aethiopicus

By

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Abstract

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Smooth muscles from the glottis sphincter and pulmonary parenchyma were excised from freshly killed specimens of the African lungfish, *Protopterus aethiopicus*, and mounted in a bath of Ringer solution. While responses to pharmacological agents were studied. Both muscle preparations gave qualitatively similar responses. Acetylcholine caused contraction, which was abolished by pretreatment with atropine. Histamine and serotonin also contracted the muscles, their effect being diminished by mepyramine and methysergide. Adrenaline and noradrenaline [illegible] the muscle preparations. Neither reserpine nor propranolol affected this response. Isoproterenol had slight relaxing effect. Anoxia and hypercapnia did not influence the contractile state of the muscles.

The results obtained are discussed in relation to the response of mammalian tracheobronchial muscles to the same pharmacological agents.

During the evolution of pulmonary breathing the mechanics of lung ventilation and the efficiency of the lung in gas exchange have been profoundly altered. Marked changes have also occurred in the anatomical distribution and functional significance of the smooth muscles of the respiratory apparatus. In primitive lungs as apparent in lungfish (dipnoan) smooth muscles are richly distributed in prominent ridges and connections throughout the pulmonary parenchyma. The lungfish has no air distributing system comparable to trachea and the bronchial tree of higher tetrapods. A prominent sphincter marks the entrance to the lung from the pharyngeal floor. Information is lacking concerning the pharmacological characteristics of the smooth muscles of either sphincter or lungs.

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The present investigation deals with the responses of these structures from the African lungfish, *Protopterus aethiopicus* to pharmacological agents with well defined effects on mammalian tracheo-bronchial muscle. Agents with specific effects on the smooth muscle preparations were also injected into intact non-anesthetized fish and their effects on breathing behaviour observed.

Material and methods

10 specimens of the African lungfish, *Protopterus aethiopicus* were flown from Lake Victoria, Uganda, to Oslo, Norway. The fish varied in weight from 1.4 to 6.1 kg. The glottis sphincter and strips of lung tissues were excised from freshly killed specimens, and the preparations suspended in a bath of Ringer solution (iso-osmotic to 0.75 % NaCl) with one end anchored and the other attached to a Grass Force-Displacement transducer (FT03). The output from the transducer was recorded on a Grass Polygraph (model 5D). Drugs were added to the bath during continuous monitoring of changes in muscle tension. The preparations were washed thoroughly between each drug administration by changing the Ringer solution repeatedly. The same experimental arrangement was used to test responses to hypercapnia and anoxia, by bubbling respectively 6 % CO_2 in air or pure nitrogen through the bath. The following drugs were used: acetylcholine chloride, adrenaline chloride, noradrenaline chloride, isoproterenol chloride, histamine phosphate, serotonin creatinine sulfate, atropine sulfate, regitine mesylate, propranolol chloride, mepyramine maleate and methysergide maleate. Doses are expressed as µg of the salts.

In vivo effects were noted after intravenous injection of drugs through chronically indwelling catheters while the fish were under observation in large aquaria.

Results

In vitro effects. Fig. 1 summarizes the responses of the isolated muscle preparations to pharmacological agents.

Acetylcholine (0.1— µg/ml) gave a marked contraction of both the pulmonary smooth muscle and the glottis sphincter. The contractions were transient, and peak tension developed within 15—30 sec. The contractions were totally prevented if the muscle preparations were pre-treated with atropine (—4 µg/ml). A relaxing effect on both types of muscle preparations resulted from the administration of adrenaline and noradrenaline in concentrations of 0.5—5 µg/ml. This effect was slow to develop. It also persisted longer than the effect produced by acetylcholine and was not influenced by addition of regitine and propranolol (2 µg/ml) to the bath. Both histamine and serotonin (0.1—2 µg/ml) resulted in contraction of both muscle preparations. These effects appeared later and lasted longer than the response to acetylcholine. The effect of histamine could be prevented by mepyramine (2 µg/ml) and methysergide (1—2 µg/ml) had a diminishing effect on that of serotonin. Isoproterenol (0.5— µg/ml) either had no effect or induced a slight relaxation of the muscles.

The smooth muscle preparations did not show any response to changes in the gaseous composition of the bath within the study period (20—30 min).

In vivo effects. Only suggestive inference can be given to the intravenous drug injections on intact animal. Acetylcholine injections sometimes resulted in attempts to breathe air. Injections of histamine intravenously similarly and more ...

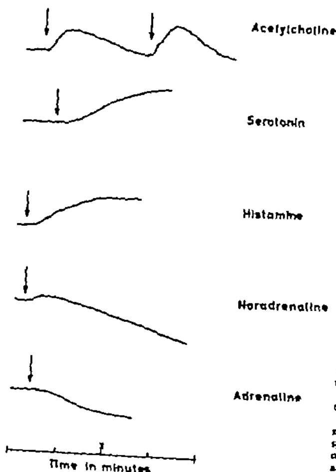


Fig. 1. Changes in tension of isolated smooth muscle preparations from glottis sphincter and lung of the lungfish, *Protopterus aethiopicus* produced by drugs added to the bath at time indicated by arrow. Upper graph obtained in smooth muscle from the glottis sphincter, the others in smooth muscle from the lung. Both preparations gave qualitatively similar responses.

resulted in an increased frequency of air breaths. Often the accelerated breathing frequency entailed a massive release of gas bubbles from the lungs, a response also typical of serotonin injections. Both histamine and serotonin injections were suggestive of additional central effects, commonly resulting in adverse behaviour, e.g. splashing about in the aquarium.

Discussion

The functional significance of the smooth musculature in the phylogenetically primitive lung in dipnoans differs *fundamentally* from that in the higher tetrapods. In mammals, the tracheo-bronchial smooth muscles exert their primary function by influencing the airway resistance and hence the distribution of air to the exchange surfaces of the alveoli. Concurrently, changes in the contractile state of the tracheo-bronchial muscles will bring about changes in the dead space of the air conducting

system. For the early lung as manifested in the lungfish, the conducting airway is extremely short (1—2 cm long in a 3 kg lungfish) and represented only by the pneumatic duct. The resulting anatomical dead space is accordingly almost non existent The mechanics of breathing are also totally different, and filling of the lung is accomplished by positive pressure breathing utilizing the buccal force pump, consisting of the muscular walls in the posterior part of the buccal cavity and pharynx, supported by a well-developed hyoid muscular apparatus (Grigg 1965) During the forced filling or swallowing of air int the lung the muscular branchial opercula remains tightly closed, and a muscula sphincter at the opening of the esophagus prevents air from entering the stomach. At this time the smooth muscles of the glottis sphincter the pneumatic duct, and the lung itself must be relaxed. Subsequent to filling, the glottis sphincter must remain tightly closed n order to prevent the inspired air from escaping Expiration is thought to occur in response to a contraction of the smooth muscles of the lung It seems important that air is expelled by excess pressure in the l ng through at least a partially contracted glottis sphincter in order to permit expiration in underwater position without adm tting w ter into the lung Recordings from the Australian lungfish, *Neoceratodus f rsteri* have confirmed that intrapulmonary pressure remains positi e throughout the entire breathing cycle (Johansen, Lenfant and Grigg 1967)

On the basis of the marked differences in the distribution and functional role of the smooth muscles in the lower and higher vertebrate lung, as pointed out above it ppears to be of considerable nterest that the smooth muscles of the lungfish lung as studied in the present work respond to drugs principally in the same way as the tracheo-bronchial musculature of mammals (Widdicombe 1963) Scant infor mation is a ailable on representatives from other vertebrate classes but the frog lung muscles re known to contract in response to acetylcholine and relax in re sponse to adrenal ne noradrenaline and serotonin (D jkstra and Noyons 1939 Brecht and Jeschke 1960) A non-specific ariable effect of serotonin on rious types of smooth muscle has been emphasized by Prosser (1960) Pharmacological studies of the muscularis mucosa of the teleost swimbladder a homologue structure t the dipnoan lung show two types of response to catecholamines adrenaline and noradrenaline contract the secretory mucosa, while the bsorbing mucosa is relaxed (Fänge 1962) Fange (1966) implies that this ndicates the presence of important differences between secretory and absorbent area of the teleost swimbladder It is reported that release of ga ia the pneumatic duct physostome teleosts is under adrenergic control while ir gulped through the duct is under cholinergic control (Fänge 1966) O r da do not permit conclusions to be drawn regarding the inner v tion of the smootl uscles f the d pnoa l ng The failure of the β-adrenergic blocking agent pro nolol t inhibit th relaxi g effect of drenaline nd nor adrenaline may suggest tha the n mon la f cation of receptors does not apply t the preparation studied

In contrast to t onformity of response t drugs in the smooth muscles of the lungfish lung and he mammalia tracheo-bronchial muscles, it merits ttention th

the dipnoan smooth muscle is seemingly unresponsive to anoxia or hypercapnia. Intact lungfish display considerable tolerance to anoxia, whereas the denervated mammalian tracheo-bronchial musculature reacts by relaxation to both anoxia, hypoxemia and hypercapnia (Nisell 1950). These responses have been considered important as local changes in air distribution may improve conditions in hypoventilated areas of the lung (Widdicombe 1963). The response difference between higher and lower forms of vertebrates to the gaseous composition in the lung can also be correlated with the presence of a specialized nutritive circulation via the bronchial arteries in mammals, whereas such circulation seems to be nonexistent in lower forms.

One final point deserves mention when comparing the role of pulmonary smooth muscles in higher and lower forms. In dipnoans, amphibians and reptiles there is only a partial anatomical separation of the systemic and vascular circuits. The two circuits will therefore be perfused under largely similar pressure conditions. It seems conceivable that the elevated intra pulmonary pressure demonstrated in the lungfish and in some species of aquatic turtle (Johansen and Hanson 1966) may play an additional role by counteracting the relatively high intra vascular pressure in the lung capillaries and thus prevent effusion of plasma into the lung. That the presence of large elements of smooth muscle in the pulmonary parenchyma of lower vertebrates possibly not only aids the mechanics of breathing is demonstrated in several species of aquatic turtles, where the lungs are profusely equipped with smooth muscle despite the presence of a negative pressure breathing brought about by the action of extra pulmonary respiratory muscles.

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Plasma Renin Activity after Bleeding in Rats with Transected or Destroyed Medulla Spinalis

By

Ljubomir Božović and Jan Castenfors

In a previous study we have found that the increase of plasma renin activity after bleeding can be prevented if the animals are pretreated with a ganglion blocking agent (Božović and Castenfors 196) Local anesthesia of the renal nerves inhibits the renin release in the majority of dogs subjected to bleeding (Bunag Page and McCubbing 1966) The increase of angiotensin concentration in blood after bleeding is also abolished by blocking the renal nerves of dogs with lignocaine (Hodge Low and Vane 1966)

To explore further the role of the nervous system in renin release in the present study w subjected rats with transected and destroyed spinal cords to bleeding

The spinal cord of female albino rats, 200— 40 g body weight, under ether anesthesia, was transected on T 6 level or destroyed caudally from this level by introducing an elastic metal wire to the end of the vertebral canal One group of the operated rats was bled hrs and one group 21 hrs after the operation Before bleeding the animals were anesthetized with sodium pentothal (5 mg 100 g body weight i p) and prepared for the bleedings as described in our previous study Božović and Castenfors 196) The blood was taken by opening the cannula in one carotid artery and letting the blood run into heparinized tube From the other carotid artery the pressure was recorded The blood sample had volume of 1.5 ml per 100 g body weight of the bled animal After 45 min second sample of blood was taken in the same w From each animal accordingly two samples of blood were obtained The volume of plasma in which renin activity was determined was 1.5— 0 ml Renin activity was measured according to modification of the method described by Boucher et al (1964) For further details see Božović and Castenfors 196

The results of plasma renin activity determination are given in Table I Statistical analysis of the data was done by the matched pairs method. The single values and means were rounded to 10 ng angiotensin per 100 ml of plasma.

In all groups mean plasma renin activity was higher after bleeding The rise was not significant in rats with spinal cord transected hrs before bleeding In this group three rats had lower level in the second sample than in the first 1 rat showed small increase and two had an increase which was higher than the error of the method In all other groups each animal showed a substantial increase of plasma renin activity after bleeding The variations of plasma renin activity were not correlated to the blood pressure changes caused by bleeding

TABLE 1 Effect of bleeding on plasma renin activity in rats with transected and destroyed spinal cord

Number of rats	Spinal cord operation and time of bleeding after operation	Plasma renin activity in ng of angiotensin/100 ml			P
		Before	After	Mean diff. ± SEM	
8	Transection — 2 hours	1 160	1 470	− 310 ± 199	> 0 1
8	Destruction — 2 hours	1,220	2,530	+ 1,310 ± 251	< 0 01
8	Transection 24 hours	2,680	13,000	+ 10,320 ± 2,877	< 0 01
8	Destruction 24 hours	2 220	6 480	+ 4,260 ± 1,052	< 0 01

These results suggests that in bleeding renin release is caused by a spinal reflex. The majority of animals with a spinal transection performed two hours before bleeding did not react with an increase of plasma renin activity. This unreactivity may be explained by the fact that these rats were in a state of spinal shock. 24 hrs after spinal transection renin release is enhanced as compared to normal animals (Božović and Castenfors 1967). This hyperactivity indicates that after 24 hrs a state of hyperreflexia is established which is supported by the observation that at this time somatic spinal reflexes were easily elicited. The fact that animals with destroyed spinal cords reacted with a significant increase of plasma renin activity suggests that in renin release a non nervous basic mechanism is also operating. A rapidly developing denervation hypersensitivity of the juxtaglomerular cells to circulating catecholamines cannot be excluded. This possibility is now being tested in our laboratory.

† Supported by grants from Forenade Liv (Group Insurance company) and the Swedish National Association against Heart and Chest Disease Stockholm Sweden

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The CSF/Blood Potential in Sustained Acidosis and Alkalosis in the Rat

By

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The stability of CSF pH in spite of marked systemic acid-base disorders as well as the existence of an electrochemical disequil brium for H and HCO^- between cere brospinal fluid (CSF) and blood plasma, ha e led to the assumption of an active transport regulation of CSF pH The hypothesis of an active transport of H or HCO between CSF and plasma was strengthened by electrochemical potential differences calculated for chronic acid base conditions (Severinghaus 1965) How ever the calculations were based on the assumption that the CSF/plasma potential in chronic acid-base changes returns to normal values after th nitial cut variation with plasma pH (*cf* Held, Fencl and Pappenheimer 1964) nd this assumption has been questioned in a recent report (Goodrich 1966)

In the present paper a report will be given of measurements of the electrical potential difference between CSF and blood n rats during sustained respiratory acidosis, and during sustained nonrespiratory acidosis and alkalosis. The procedures followed were identical to those employed previous studies from the laboratory (Siesjö and Pontén 1966 a and b) in which the CSF bicarbonate wa measured whence electrochemical potential differences could be calculated.

The rats were either exposed to 15 CO for 24 hrs, or were injected w h isotonic acid or alkaline solutions intraperitoneally for 6 hrs Siesj nd Pontén 1966 b) At he end of the exposure pe riods he animals were anesthetized h phenobarbital p 100 mg/kg) and racheotomized. The entral tail rtery as can ula ed for blood sampling

pH, P_{O_2} nd P_{CO_2} were measured on he blood samples using micro electrodes. The CSF plasm potential was measured b he external jugula ei nd the cisterna magna by introducing polyethylene be filled h 1 M KCl in 2 % aga in th ein, nd by piercing the tla o-occipital membrane w h m l h filled glass micropipett The KCl bridges were connected high resistance differen l plifier ia alomel referenc lectrodes

The esults of the me su ment given n he figure The mean CSF plasma potential in the con l g oup given art ficial t acellula fluid p wa 3 3 ± 0 4 mV at a mean plasma pH of 7 4 In the lkalot gro p the correspond g values were − 1 8 ± 0 V t mean pH of 7 54 and in the acidotic groups the mean values were 7 3 0 7 mV nonrespiratory acidosis and 9.5 ± 0 2 mV respiratory acidosis) at mea pla m pH lues of 7 26 and 7 19 respecti ely The results clearly show that ma ked ariation in the CSF plasma potential re upheld in

different mechanism. Their action is not blocked by enzyme inhibitors (Högberg and Uvnäs 1960, Moran, Uvnäs and Westerholm 1962). Histamine release may also be brought about in a simple way by osmotic disruption of the cells in hypotonic salt solutions (Fawcett 1955).

The purpose of the present investigation was to establish if those structural features observed in rat peritoneal mast cells after treatment with compound 48/80 could be induced by other liberating agents, and to compare the effects on mast cells of liberators with different modes of action.

Materials and methods

Preparation of cells and incubation technique

Peritoneal cells were obtained from male Sprague-Dawley rats (350—450 g) by peritoneal infusion with a salt solution containing NaCl, 154 mM; KCl, 2.7 mM; $CaCl_2$, 0.9 mM; human serum albumin, 1 mg/ml and buffered with 10 per cent (v/v) Sörensen phosphate buffer (Na_2HPO_4 + KH_2PO_4, 6.7 × 10 M) pH 7 as described previously (Bloom and Haegermark 1963). After removal from the peritoneal cavities, the cell suspensions were centrifuged at 350 × g for 3 min. The cells were then resuspended in the buffered salt solution and the cells from the different rats were pooled. The number of animals in each experiment was chosen so that cells corresponding to one rat could approximately be divided into 2—3 incubation tubes. Aliquots (2.5 ml) of the pooled cells were added to centrifuge tubes containing 2.5 ml of the histamine liberating agents dissolved in buffered salt solution. However, when the effect of hypotonicity was studied, 1 ml of cell suspension was added to the tubes containing various volumes of distilled water (0—9 ml) to give the appropriate final dilution of the salt solution. The cells were incubated in duplicate samples at 37° C for 10 min, after which the samples were centrifuged for 5 min at 350 × g. One of each duplicate sediment was used for morphological studies, while the other was resuspended in buffered salt solution and heated in boiling water for 5 min to extract histamine, which was then determined. Histamine content was measured in all supernatants.

Histamine assay

Histamine was determined on the atropinized guinea-pig ileum in the initial experiments, while the more convenient fluorometric assay described by Shore *et al.* (1959) was used later in the investigation.

Preparation for morphological studies

The sediments to be studied in the light and electron microscope were resuspended in glutaraldehyde, rinsed and post fixed in osmium tetroxide and then embedded in Epon. The preparatory procedures have been reported in detail in a previous paper (Bloom and Haegermark 1965). For light microscopy thick, 1 µ sections were made from the Epon blocks, placed on microscope slides and were then stained in 1 per cent toluidine blue 30—60 sec. The sections were mounted in DPX and studied under oil immersion.

For electron microscopy thin sections were made on an LKB ultrotome. The sections were stained with uranyl acetate and lead citrate and were examined with a Philips EM 200 electron microscope. Electron micrographs were taken at an original magnification of 1,200—30,000.

Materials

Bee venom, purified on Amberlite IRC-50, was prepared and kindly supplied by Dr B. Högberg, AB Leo, Hälsingborg, Sweden. Human serum albumin, AB Kabi, Strängnäs, Sweden.

Results

Histamine release

In Table I—III are shown the data on histamine release induced by different concentrations of bee venom, n-decylamine and by varying degrees of hypotonicity. Clear dose-response relationships were obtained. Increase of the bee venom concentrations 25 times, from 0.2 to 5.0 µg/ml, raised the histamine release from 69

to 72 per cent, whereas a 2.5-fold increase of the decylamine concentration from 20 to 50 μg/ml, was sufficient to increase the release from 5 7 to 93 per cent. By dilution of the physiological salt solution to one third of its original concentration a slight histamine release (5 6 per cent) was elicited. By further decreasing the osmolarity e.g dilution 3 75- and 5-fold, 21 and 91 per cent histamine was released, respectively

Morphological observations

Normal mast cells

Numerous mast cells were observed in all controls. Under light microscopy these cells exhibited normal characteristics. The cells were generally filled with granules which stained blue or deep purple with toluidine blue. In many cells a few granules deviated in tinctorial properties and showed up in a clear red or pinkish hue The latter type of granule was sometimes observed to lie within vacuole like cellular structures.

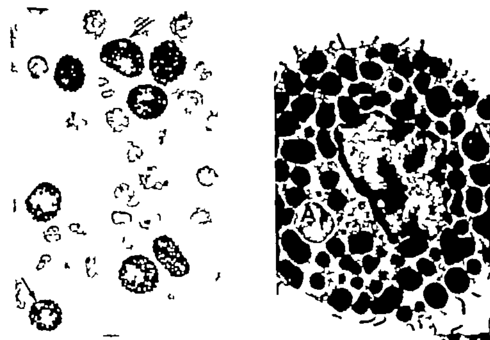


Fig 1 Light micrograph of normal ra peritoneal cells toluidine blue stained Epon section The mast cells are easily recognized by their intensely stained granular contents. Arrows indicat occasional altered granules, visible in the light microscope 1,000.

Fig 2. Low power electron micrograph of normal rat peritoneal mast cell. Fingerlike cy oplasmic protrusions are seen at he cell periphery and the majority of the mast granules exhibit fairly electron dense homogeneous ppearance Tw granules undergoing alteration are also observed (A) 8,400

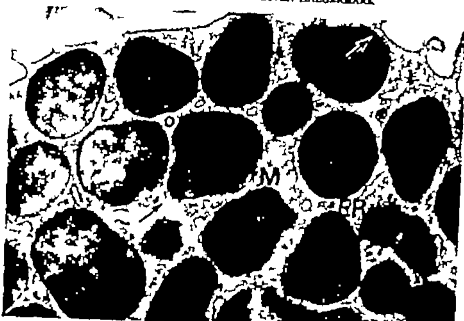


Fig. 3. Portion of cytoplasm from normal rat peritoneal mast cell. Here mitochondria (M) and endoplasmic reticulum (ER) can be seen, as well as the distinct membranes which delimit mast granules from the cytoplasm proper (arrows). Electron micrograph × 30,000.

The fine structure of normal rat peritoneal mast cells has been described in detail in a recent paper (Bloom and Haegermark 1965). The electron microscope makes it possible to distinguish between two major types of mast cell granules, one type dominates the granule population of normal peritoneal mast cells, is homogeneous, electron dense and surrounded by a distinct membrane which may be separated from the granule matrix by an extremely narrow electron lucid space. The other type of granule appears to be somewhat larger, is less electron dense, shows a fine granular or filamentous substructure and is generally separated from its surrounding membrane by a space of varying width. The observations of transitional forms between these two types of granules led us to assume in our previous investigation that the latter type merely represent normal granules that have been "altered" in some way. Normal peritoneal mast cells from controls are seen in Fig. 1—3.

Mast cells exposed to bee venom

Mast cell appearance was normal in specimens treated with the lowest dose of bee venom (0.1 μg/ml—histamine release 4.4 per cent). After incubation with 0.2 μg/ml of bee venom (histamine release 6.9 per cent) there appeared to be a slight increase in the number of mast cells showing cellular changes. The latter were very similar to those observed in cells exposed to small doses of compound 48/80 (Bloom and Haegermark 1965). Granules undergoing alteration exhibited a slight increase in size, a widened space between granule matrix and surrounding perigranular mem-

TABLE I. Histamine release induced by bee venom.
One of each duplicat sediment (— in the table) was prepared for electron microscopy

Bee venom µg/ml	µg Histamine			Per cent release
	Sediment	Supernatant	Total	
0	—	0.75		
	17 15	0.70	17.85	3.9
0.10	—	0.93		
	19.50	0.90	20.40	4.4
0 20	—	1.33		
	17.50	1.30	18.80	6.9
0 50	—	4.93		
	13.10	4.75	17.85	27
5 0	—	13.90		
	5.30	13 60	18.90	72
50.0	—	20.45		
	0.70	21.35	22.05	97

brane and a decrease in electron density The latter feature was accompanied by the appearance of a delicate reticular or finely granular texture of the matrix which was almost entirely masked in normal, unaltered granules. Occasionally there was evi dence of a fusion and subsequent disintegration of closely opposed perigranular membranes resulting in the formation of a vacuole containing two or more altered granules. In these specimens signs of peripheral granule release were rare, as were single, altered granules located extracellularly It should be pointed out that a large portion of the mast cells appeared perfectly normal and showed only 1—3 altered granules in a cross-section of the cells.

In mast cells exposed to 0.5 µg/ml of bee venom (histamine release 27 per cent) signs of granule release were common, and numerous altered granules appeared throughout the cytoplasm of many cells (Fig 4) Specimens treated with 5 µg/ml of this releaser (histamine release 72 per cent) showed all mast cells encountered to be affected to some degree and in the process of releasing numerous granules (Fig 5) However in some cases perfectly normal granules surrounded by a distinct perigranular membrane were observed in these cells. Numerous extracellular granules, all of the altered type were encountered throughout th specimen

With the highest dose of bee venom employed (50 µg/ml—histamine release 97 per cent) no mast cells with intact cell membrane were observed. All that remained of these cells were the nuclei surrounded by a thin rim of broken up cytoplasm in which swollen and distorted mitochondria could be recognized, as well as fragments of the rough-surfaced endoplasmic reticulum. Large numbers of altered mast granules surrounded these cell remains No unaltered ones were observed in any of the sections studied in the electron microscope

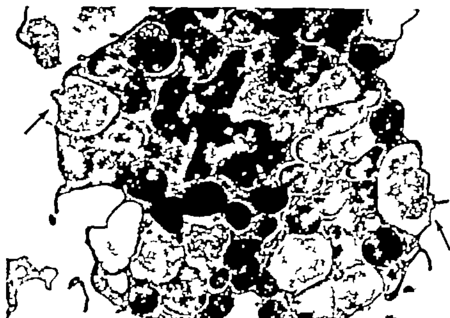


Fig. 4. Rat peritoneal mast cell after incubation with 0.5 μg/ml of bee venom. Numerous mast granules are undergoing alteration. At the periphery of the cells several vacuoles containing altered granules are present, some are opening up to expel their contents (arrows). Electron micrograph. × 9,500.



Fig. 5. Rat peritoneal mast cell exposed to 5 μg/ml of bee venom. Perigranular membranes have fused and disintegrated and large peripheral vacuoles containing several altered mast granules has formed. Note the presence of single, unaltered granules (arrows). Electron micrograph. × 16,000.

TABLE II Histamine release induced by n-decylamine
One of each duplicat sediment (— in the table) was prepa ed for electron microscopy

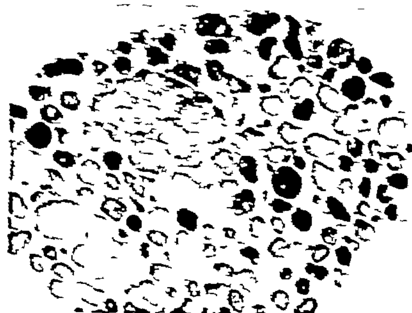
Decylamine µg ml	µg Histamine			Per cent release
	Sediment	Supernatan	Total	
0	—	0.7		
	17 15	0 70	17.85	3.9
5	—	0.70		
	20 00	0 70	20.70	3 4
10	—	0.70		
	20 00	0.70	20.70	3.4
20	—	1.20		
	19 00	1 15	20.1	5 7
30	—	6 20		
	15.20	5.25	20.45	26
50	—	17 50		
	1.30	18.00	19 30	93

Mast cells exposed to n-decylamine

The effects of decylamine on mast cell morphology were found to differ from those obtained with bee venom When incubated with decylamine at the lowest concentra tions used in these experiments (5 10 and 20 µg ml histamine release 3 4 and 5.7 per cent) most mast cells appeared perfectly normal A few cells, however exhibited certain characteristic features In these the cytoplasmic perigranular membran system appeared fragmented, and within fairly large areas such membranes seemed to have disintegrated (Fig 6) Altered and unaltered granules appeared randomly distributed throughout the cytoplasm In these affected cells even the cell membrane was disrupted n various location Mitochondria appeared swollen and th sacs of the rough-surfaced endopla mic reticulum appeared widened However in spite of such changes the cells ppeared to maintai some form of integrity as they appa rently did not break up and dispel their content

Incubation with 30 µg ml of decylamine histamine release 26 per cent resulted in rather widespread effect on the mast ll Many cells appeared affected in the same manner a recently described Fig 7 Extracellular gran les of altered type were rather frequent, a wer gr oups of gran les surrounded by remnants of a plasma membrane cytoplasmi membr nes and m tochondria In such groups both altered and unaltered granules wer observed Cross sections of mast cell which had an tact cell membrane showed granules in various stages of alteration

W th the highest dose of decylam employed 50 µg ml—histamine release 93 per cent no normal-appea ing ntac ma ell were observed. However many of the mast cells till appeared un alth ll membranes were disrupted and had to large extent, disin egrated All mast granules appeared altered to some ex ent and mo t of them were grossly ltered Cytoplasmic esicles and membrane fragments wer often found randomly attached both to solated granules and granule lusters.



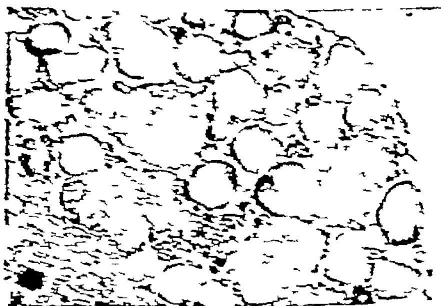


TABLE III. Histamine release induced by hypotonic salt solutions.
One of each duplicate sediment (— in the table) was prepared for electron microscopy

Dilution of isotonic salt solution	µg Histamine			Per cent release
	Sediment	Supernatant	Total	
1	—	0.2		
(undiluted)	16.5	0.2	16.7	1_
1.5	—	0.3		
	18.1	0.3	18.4	1.6
3 0	—	0.8		
	13.5	0.8	14.3	5 6
3.75	—	3.1		
	11.9	3.2	15.1	1
5 0	—	13.1		
	1.5	12.8	14.3	91
7.5	—	14 8		
	0.7	14 4	15.1	95

Mast cells xposed to hypotonic salt solutions

The morphological changes which appeared in mast cells after exposure to hypotonic solutions showed certain similarities to those seen after treatment with a-decylamine. The earliest detectable effects of decreasing osmolarity on peritoneal mast cells were observable when the buffered salt solution was diluted 3-fold histamine release 5 6 per cent) In the light, as well as the electron microscope, the majority of the cells appeared normal. However a number f cells contained an increased number of altered granules, and some cells were disrupted. In undisrupted but apparently affected cells, the changes could be summarized as follows see Fig 8 The cell membrane appeared intact in cross section. The intergranular cytoplasm was electron lucid and the sacs of the endoplasmic reticulum appeared distended and filled with electron dense material. Widely spread intracellular granules in arious stages of alteration were seen, as were perfectl normal ones.

At 3 75-fold dilution of the salt solution histamine release 21 per cent many more cells were affected, while the cellular changes were still of the same general character as those described abov Fig 9 While some cells appeared intact and only slightly affected, numerous mast cells were totally disrupted and only an accumulation of granules marked the remains of cell. The most striking finding was the large number of unaltered granules freely exposed to the extracellular medium Such granules were mostl surrounded by clearly visible perigranular membranes while altered ones inevitabl lacked this structure.

Increased dilution 5- and 7 5-fold, histamine release 91 and 95 per cent respec tively led to total disruption f all mast ells. At a 5-fold dilution a few per cen of the extracellular granules were membrane-enclosed and apparently unaltered Unaltered granules were rare at the highest dilution.

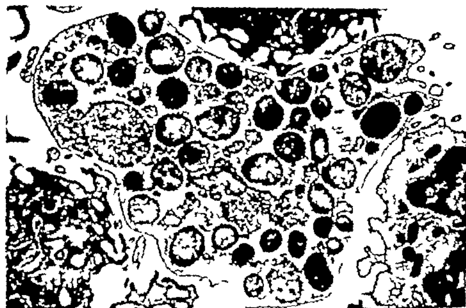


Fig. 8 Ma ll ed ith hypotonic salt solution (3 dil ted The cell membrane appears intact bu h oplasm is electron lucid nd the mast granules are idely separated. While mostly nal ered and membrane-delimi ed some granules re non-delimited nd altered. There are no signs of peripheral gra ule le se. Elect on micrograph 8 7 0



Fig. 9 Rat peritoneal mas ell trea ed h h potonic salt solution (× 3 75 diluted). Although the plasma membrane is no longer intact ma y gr nules re membrane-delimited and unaltered. Gross changes have taken plac he in granula ytoplasm hich apart from distended tu of the endoplasmic reticul ppears mor or less empty Electron micrograph. × 10,000.

Discussion

The ultrastructural changes in rat peritoneal mast cells caused by increasing doses of the histamine liberator compound 48/80, have recently been described in detail (Bloom and Haegermark 1965) When comparing the effects of the liberating agents employed in the present investigation with the features induced by compound 48/80, it was found that bee venom induced similar changes in mast cell morphology as compound 48/80 while n-decylamine and hyposmosis did not.

The same characteristic sequence of events take place in mast cells exposed to increasing concentrations of bee venom as has been described for cells treated with compound 48/80 This sequence involves alteration of mast granules, formation of granule-containing vacuoles and finally expulsion of one or several non-delimited, altered granules. During this process the cytoplasm is delimited from the granule containing compartments by the so-called perigranular membranes. Although the electron micrograps are only static pictures of events that have ceased at various stages, the sum of events observed gives the impression of an active process taking place.

Bee venom has been found to release histamine in a way very similar to compound 48/80. It was earlier thought that this activity could be due to phosphotidase A which is a component of bee venom. However recent studies indicate that the histamine releasing activity of bee venom. is found in a fraction other than that containing phosphatidase A (Rothschild 1965 Fredholm 1966, Fredholm and Haegermark 1967) It is possible that this fraction as well as the polymer amine 48/80 triggers a cellular enzyme mechanism which in turn leads to release of histamine. The similarities in ultrastructural changes which take place during histamine release by compound 48/80 and bee venom support the hypothesis of a similar release mechanism.

Decylamine and hyposmosis, on the other hand, lead to structural changes of a different type. There is a more general effect on the cells, and especially on cell and cytoplasmic membranes. The ultimate result is a granule release by a deterioration of the cells and granule-containing compartments rather than by a seemingly dynamic expulsion of these structures. It lies close at hand to attribut the observed structural changes to the surface active properties of decylamine, and the disruptive activity of solutions with deceased osmolarity It is interesting to note that the mast cells appear to be exceptionally sensitive to these factors since other cells of the peritoneal fluid g leukocytes, are always much less ffected. This is in agreement with the observations by Fawcett (1955) that mast cells in the rat mesentery were more fragile to hyposmosis than other tissue cells. As the mast granules are known to contain proteolytic enzymes (Gomori 1953, Benditt and Arase 1958, and others) t is possible that these substances contribut to the internal cellular breakdown.

Histamine is only loosely bound n the mast granules, probably by simpl ionic bonds (Uvnäs 1964 Lagunoff *et al* 1964 and others) Simple cations such as N and K have been shown t release histamine from mast granules solated in ion free media However our knowledge of the factors involved in histamine release



Fig 10 Rat peritoneal mast cell after exposure to a sal solution diluted 5 fold All that remains of the cell a umber of free non-delimited, altered mast granules which surround the remnants of the cell ncleus. Electron micrograph × 10,500.

from whole mast cells exposed to liberators is scanty Intracellular mast granules are always separated from the cytoplasm proper by a membrane As histamine may be released by concentrations of cations well within the range expected intracellularly it has been proposed that the perigranular membrane serves to isolate the mast granule from the ionic milieu of the cell sap (Lagunoff 1966) If this is the case t follows that permeability changes in the cell and cytoplasmic membranes would enhance the possibilities for extracellular as well as intracellular cations to react with the mast granules The morphological changes after treatment with compound 48/80 (S ngleton and Clark 1965 Bloom and Haegermark 1965, and others) and bee venom would agree with a disturbed balance between cytoplasm and granular compartments the mast granules appear to swell, lose their dense structure and become surrounded by a halo It must be stressed that although the decreased electron density of the granules is observed in connection with histamine release from the cells, this observation does not necessarily imply that the granule alteration *per se* directly effects the release of this amine from its granular binding sites. As pointed out earlier (Bloom and Haegermark 1965) the changes observed may also be the result of other processes accompanying release of histamine from the cells.

It would furthermore be presumptuous to draw definite conclusions from the present findings as to whether or not histamine release from mast cells requires the actual extrusion of mast granules from the cells as has been postulated (Uvnäs and Thon 1966) Some recent evidence in favour of amine release from mast cells without

concomitant granule release has, however been presented by Ritzén (1966). Using microspectrofluorometry he observed formation of peripheral vacuoles devoid of 5-hydroxytryptamine in rat peritoneal mast cells treated with compound 48/80. Staining the same cells with toluidine blue releaved the presence of metachromatic granules and substance in many of these vacuoles.

It is also interesting to note that the mast granules which are released by compound 48/80 and bee venom are all of altered type and are not membrane-delimited. With decylamine and hypoosmotic salt solutions the situation is different. As the mast cells disintegrate numerous granules of both altered and unaltered type may be found extracellularly. In this case the unaltered ones are mostly surrounded by a visible perigranular membrane.

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Studies on the Time Course of Histamine Release and Morphological Changes Induced by Histamine Liberators in Rat Peritoneal Mast Cells

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Abstract

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The histamine release process in rat peritoneal mast cells, exposed to histamine liberators, can be terminated by the addition of ice-cold salt solution. This was taken advantage of for studies on the time course of histamine release and concomitant morphological changes induced by compound 48/80 bee venom and n-decylamine After addition of compound 48/80 or bee venom, histamine was released in about 10 sec at 37 C and about 30 sec at 22° C, independently of the liberator concentration. At 22 C the release was preceded by a lag period which was shorter with higher liberator concentrations The enzyme inhibitors potassium cyanide and N-ethylmaleimide did not alter the duration of the reaction induced by compound 48/80 or bee venom, although they lowered the final release The release curve showed a different shape when histamine was released by n-decylamine The morphological changes, studied both in the light and electron microscope, were found to be correlated to the histamine release.

Several investigators have studied the time course of histamine release using different techniques and organs Among the early reports is that of Schild (1939) who found that the release from guinea pig aorta was a rapid process, taking place within the first minutes after contact between releasing agent and the tissue. From studies on the blood pressure response caused by released histamine in cats, Paton (1956) concluded that the release process must take only a few seconds or even less. However it is difficult to determine the actual time course of the release from cellular stores, i.e. from the mast cells, when whole animals, tissues or tissue slices are studied, as diffusion must be considered This difficulty may be reduced by studying free cells in suspension

When rat peritoneal mast cells in suspension are exposed to a histamine releasing agent, they have been shown to undergo rapid morphological changes, "degranula-

tion" (Mota 1960 Uvnäs and Thon 1961 Keller 1961) and to release their histamine within 20 sec at room temperature (Moran, Uvnäs and Westerholm 1962) However it is not known how the time course of the release is influenced by varying the experimental conditions, and nearly all information on the release process is based on experiments in which the final degranulation or release has been studied. Such experiments have shown that many liberators, *e.g.* compound 48/80 or bee venom, evidently activate energy-requiring release processes in the mast cells (Junqueira and Beiguelman 1955 Högberg and Uvnäs 1957 Mota and Ishii 1960) while the release mechanism seems to be different for some other agents, *e.g.* decylamine (Högberg and Uvnäs 1960)

It was believed that additional information on the process of histamine release could be obtained by studying under various conditions, the histamine release and the morphological changes taking place during the very first period after contact between suspended mast cells and histamine releasers of different kinds. Compound 48/80, bee venom and *n*-decylamine were chosen for this purpose.

Methods and materials

Preparation of cell suspension

Peritoneal cells were obtained from male Sprague-Dawley rats (300—400 g) as described in detail previously (Bloom and Haegermark 1965) As suspending medium for the cells salt solution was used, containing 154 mM NaCl, 2.7 mM KCl, 0.9 mM $CaCl_2$, buffered with 10 per cent v/v Sörensen phosphate buffer 67 mM Na_2HPO_4 + KH_2PO_4 mixed in appropriate proportion to give pH of 7.0. To this solution was added 1 mg human serum albumin per ml. Cells from 2—3 rats were pooled in those experiments where only histamine release was recorded, and 7—8 rats were used in each experiment when mast cell structure was studied as well.

Incubation technique

The incubations were performed in 15 ml conical centrifuge tubes. A solution of the histamine releasing agent (0.1 ml) was first pipetted into the tubes. Thereafter 0.9 ml of the cell suspension was rapidly added to each tube. After certain intervals, the incubation was terminated by rapidly pipetting 9 ml of ice-cold, buffered salt solution into the tubes. This instantaneously lowered the temperature to a level where no release occurs and furthermore the liberator was diluted to subliminal concentration. The tubes were kept in ice water until centrifuged at 350 × *g* at 1—2° C for 10 min. The supernatant containing released histamine was carefully decanted. The histamine remaining in the cells was extracted by suspending the sediment in buffered salt solution and heating in a boiling water bath for 3 min. Histamine was determined in both the sediment and the supernatant, and the release was calculated as per cent of the total histamine content in each sample

The suspensions were incubated with the liberating agents for periods between 5 and 60 sec and in some cases for 10 min. Five sec was the shortest period during which release could be accurately measured. The "0-sec" value was obtained by adding the terminating ice-cold salt solution prior to adding the cells. Spontaneous release, measured in each experiment, was of the same magnitude as the 0-sec values

All incubations were run in duplicates. In the experiments where a parallel study of the cell morphology was made, one of each duplicate sediment was prepared for light and electron microscopy while the other was treated as mentioned above for extraction of histamine. The histamine was measured in all supernatants, also in those corresponding to the cells examined morphologically

Histamine assay

Histamine was measured fluorometrically according to the method of Shore, Burkhalter and Cohn (1959) but the purification steps were omitted and *o*-phthalaldehyde (OPT) was added directly to the samples after alkalinization (Fredholm and Haegermark 1967)

Cell preparations for light and electron microscopy

Sediments of centrifuged peritoneal cells were fixed in 4 per cent glutaraldehyde in phosphate buffer (pH 7.4). After rinsing in buffer the specimens were postfixed in osmium tetroxide, dehydrated in a series of alcohols and embedded in Epon 812. Sectioning was carried out on LKB ultrotome. Thick sections (1—2 μ) were prepared for light microscopy and stained with toluidine blue. Thin sections for electron microscopy were stained with uranyl acetate and lead citrate and were examined with Philips EM 200 electron microscope. For further details of the preparatory procedures see Bloom and Haegermark (1965).

Microscopical in vitro studies

A 0.5 μl sample of peritoneal cell suspension, prepared as described, was placed under a drop of liquid paraffin on a microscope slide and the cells were studied in an inverted microscope at a magnification of 480 x. With the aid of a micromanipulator the tip of a micropipette containing 0.3 μl of the liberator was directed near a mast cell, and the solution was rapidly injected. The time from injection until the degranulation started was recorded as, well as the duration of the process.

Materials

Human serum (bovine) (AB Kabi, Strängnäs, Sweden). Bee venom, prepared with ion exchange chromatography on Amberlit IRC-50 and compound 48/80 were kindly supplied by Dr. B. Högberg, AB Leo, Hälsingborg, Sweden.

Results

Light microscopical observations on mast cells in vitro

In a series of experiments the effect of compound 48/80 on mast cells was followed in the light microscope. At 22° C it was found that when compound 48/80 was added to the cell suspension in concentrations of 0.1, 1.0 or 10 μg/ml intracellular movements and expulsion of granules began after 9, 7 and 4 sec, respectively. The duration of the process was difficult to determine as there was no well-defined end-point. However, it was estimated to be within the range 15—30 sec for 0.1 μg/ml and to be slightly shorter for the higher concentrations. In cells exposed at 37° C to 0.1 μg 48/80 ml both lag period (1—2 sec) and duration (6—8 sec) were significantly shorter.

Histamine release

The velocity of the histamine release was found to increase with increasing temperature for all three liberators studied (Fig. 1—3). The curves for compound 48/80 and bee venom had many characteristics in common. The time required for the release process to be completed was not influenced by the concentration of these releasers. When the cells were incubated at 22° C with concentrations of those agents giving a final release of around 30 per cent there was a delay of 10 sec before any measurable release started (Fig. 1 a and 2 a). No delay was observed at 37° C even at the lowest concentrations studied. At 22° C maximal histamine release was generally attained after 30 sec incubation, while at 37° C only about 10 sec was required.

The time curves had another appearance when histamine release was induced by n-decylamine (Fig. 3). The most pronounced difference when compared to the other liberators, appeared with 30 μg ml, where practically no release occurred during the first 60 sec at 22° C, while at 37° C there was a considerable release. This did not reach a final level but continued throughout the incubation period. When



Fig. 1. Time course of histamine release induced by compound 48/80) 0.1 µg/ml, b) 0.3 µg/ml and) 0.5 µg/ml. Incubation temperature 37° C, ●——● 22° C ▲——▲. Cells from duplicate samples in the experiment shown in Fig. 1 b were subjected to morphological studies in the light and electron microscope (cf. Fig. 6)

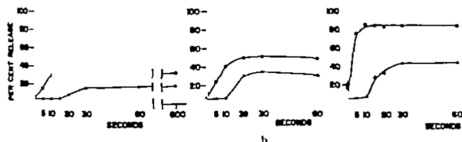


Fig. 2. Time course of histamine release induced by bee venom,) 0.5 µg/ml, b) 1 µg/ml,) 5 µg/ml. Incubation temperature 37° C, ●——● 22° C, ▲——▲. Cells from duplicate samples in the experiment of Fig. 2 were subjected to morphological studies in the light and electron microscope (cf. Fig. 7)

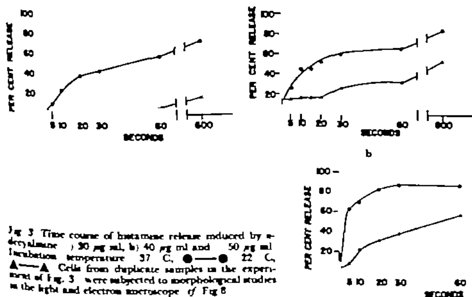


Fig. 3. Time course of histamine release induced by n-decylamine) 30 µg/ml, b) 40 µg/ml and 50 µg/ml. Incubation temperature 37° C, ●——● 22° C, ▲——▲. Cells from duplicate samples in the experiment of Fig. 3 were subjected to morphological studies in the light and electron microscope cf. Fig. 8

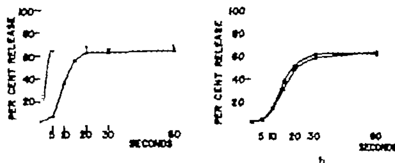


Fig. 4. Different modes of terminating histamine release induced by compound 48/80, 0.3 µg/ml. Incubation temperature 22° C. a) Incubation "terminated" by adding salt solution of 22° C, ○——○ diluting the liberator to a noneffective concentration without cooling. b) Incubation terminated by adding a salt solution of 0° C containing compound 48/80, 0.3 µg/ml ●——● and b) Incubation terminated by combination of cooling and dilution, ▲——▲

increasing the concentration of decylamine to 40 µg/ml, the release at 22° C started after a delay of 20 sec. When incubated with 50 µg/ml at 22° C some histamine release was observed already after 5 sec.

Since the incubations were routinely stopped by a combined effect of dilution of liberator and decrease of temperature, the following experiments were carried out to establish if one of these factors was predominant in arresting the release process. Cells were incubated with compound 48/80 at 22° C. To obtain rapid dilution of the liberator without cooling, salt solution of 22° C—instead of 0° C—was added. To accomplish rapid cooling without changing the 48/80 concentration, ice-cold salt solution containing this compound was added. As a control in both cases the reactions were stopped in the usual way by diluting and cooling. It was found that dilution alone did not stop the reaction, which continued to completion (Fig. 4 a). On the other hand cooling alone had the same terminating effect as the combination of dilution and cooling (Fig. 4 b).

It has been shown that the amounts of histamine released from mast cells by compound 48/80 and bee venom may be diminished by various enzyme inhibitors (Uvnäs and Thon 1961, Fredholm and Haegermark 1967). However, it is not known if these inhibitors also influence the velocity of the release process. In this investigation the effect of N-ethylmaleimide (NEM) and potassium cyanide was studied with concentrations of the inhibitors selected to give a partial inhibition of the final release. Cells were preincubated with inhibitor for 30 min at 37° C before exposure to 48/80 or bee venom. The results from an experiment with NEM and bee venom

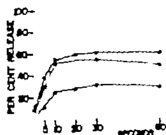


Fig. 5. The influence of N-ethylmaleimide (NEM) on the time course of histamine release induced by bee venom, 1 µg/ml, at 37° C. Without inhibitor ●——●, NEM, 7.0 × 10⁻⁴ M, ○——○. NEM 3.5 × 10⁻ M, ●——●

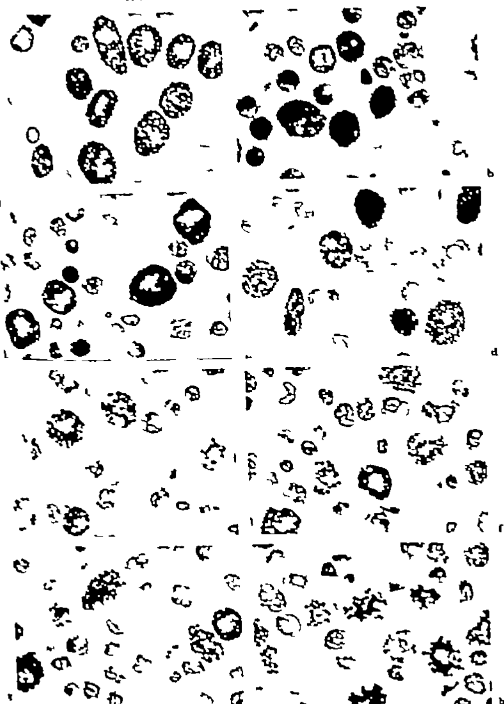


Fig. 6. Light micrographs of Epon sections stained [illegible] with toluidine blue from the experiment with compound 48/80 (0.5 μg/ml) depicted [illegible] specimens correspond to the following points on the curves (histamine release shown in parentheses): [illegible] C, 0 sec [illegible] b [illegible] 3 sec [illegible] C, 10 sec [illegible] d [illegible] 1 sec [illegible] 3 [illegible] 22 C [illegible] sec 48 [illegible] 9 sec 34 [illegible] 37 C, 5 sec 43 and h 3 [illegible] 5 sec 59 [illegible] 1000

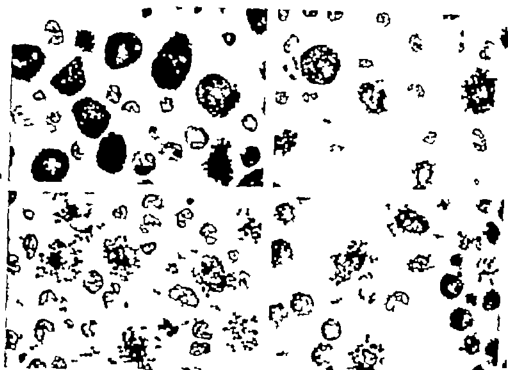


Fig 7 Light micrographs of Epon sections stained with toluidine blue from the experiment with bee venom (5 µg/ml) shown in Fig 2 Specimens correspond to the following points on the curves (histamine release values given in parenthesis) a) 22 C, 10 sec (8) b) 22 C, 30 sec (45) c) 37 C, 10 sec (86) and d) 37 C, 30 sec (84) × 1,000

are shown in Fig 5 It was found that the time until the curve levelled off was not changed by the inhibitor even though the final level of release was lowered. It was also observed that there was no lag period.

Morphological observations on fixed cells

In an attempt to catch the morphological events accompanying the release of histamine a series of experiments were performed where both the cell morphology and the amount of histamine released were studied in the same experiment. One concentration of each liberator was selected for this purpose

In order to obtain a survey of the morphological response in a large number of cells, thick sections of the specimens embedded in Epon 812 for electron microscopy were stained with toluidine blue and studied in the light microscope. Fig 6–8 show light micrographs of the cells corresponding to different points in the time curves. It was found that the mast cell changes increased with increasing histamine release and that the majority of the cells in each picture were affected to about the same extent.

Normal mast cell fine structure is shown in Fig 9 Electron micrographs were also made from specimens corresponding to each point of the time curves shown in Fig 1b 2c and 3c. The changes following treatment with compound 48/80 and bee

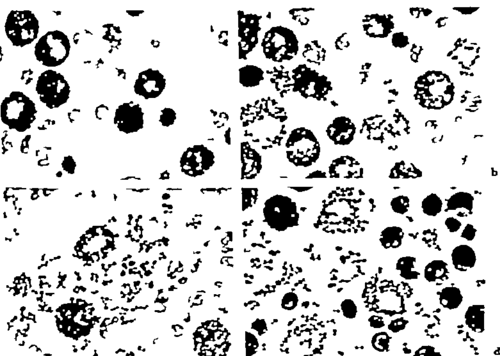


Fig. 8 Light micrographs of Epon sections stained with toluidine blue from experiment with a-decylamine (50 μg ml) shown in Fig. 3 c. Specimens correspond to the following points on the curves (histamine release values given in parenthesis)) 22 C, 5 sec (7) b) 22 C, 30 sec (38 ,)) 37° C, 5 sec (62) and d) 37° C, 30 sec (86) × 1,000

enom showed great similarities, and were of the same type as previously described (Bloom and Haegermark 1965, 1967) They involved primarily the mast cell granules which became increasingly less electron dense and seemed to be swollen. At the same time they were separated from the perigranular membrane by an electron lucid space of increasing width leaving the altered granule in a vacuole-like structure (Fig. 10) Sometimes the membranes between the vacuoles seemed to have disinte grated and larger cavities were formed containing two or more granules (Fig. 11) There were also signs of granule release, i.e. some of the vacuoles lying close to th cell membrane caused the latter to bulge and at places the membrane opened up, whereby the contents of the acuole were released to the surroundings (Fig 10—11) With compound 48/80 and bee venom no morphological changes occurred during the lag period when no histamine was released, and when the time curve for histamine release had levelled off no further morphological changes were seen.

The effect of decylamine on mast cell morphology was found to differ from that obtained with compound 48/80 or bee venom Fig 12 cf Bloom and Haegermark 1967) The changes which paralleled histamine release occurred randomly throughout the cells. Perigranular membranes were often disrupted and difficult to visualize. Mitochondria were abnormal and the sacs of the endoplasmic reticulum, when not fragmented, were widely distended Frequently the plasma membrane showed



Fig. 11 Rat peritoneal mast cell incubated at 22° C with 5 μg/ml of bee venom for 20 sec. Histamine release 30%. The central cytoplasm appears intact while at the periphery of the cell, vacuolar structures, containing one or more characteristically altered granules and with great similarities to those observed after treatment with 48/80 are observed. Several of the vacuoles are expelling their granular contents. Extracellular altered granules are also seen. Electron micrograph. × 9,500.

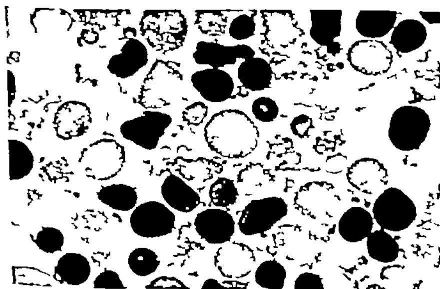


Fig. 12 Electron micrograph of a rat peritoneal mast cell incubated for 20 sec at 22° C with 50 μg/ml of n-decylamine. Histamine release 31%. Altered and unaltered granules are randomly scattered throughout the cytoplasm and vacuolar granule containing structures are lacking. The intergranular cytoplasm is greatly affected and only remnants of cytoplasmic membranes and organelles are seen. Furthermore the cell membrane is disrupted and the cytoplasmic structures appear to be freely exposed to the extracellular medium. Compare with the two previous micrographs. × 10,200.

Discussion

In this study we adopted a method, commonly used in enzyme studies designed to cause an instantaneous termination of the release reaction. The technique gave highly reproducible results down to periods as short as 5 sec. The results are in good agreement with previous reports on the time course of histamine release from rat peritoneal mast cells. For instance, when mast cells were perfused with a solution containing compound 48/80 at room temperature, the release was estimated to start within 6 sec (Uvnäs 1963) and to be completed within 20 sec (Moran, Uvnäs and Westerholm 1962). However the effects on the time course of varying the incubation conditions were not investigated.

The release curves for compound 48/80 and bee venom found in the present investigation showed many features in common whereas the time course had a somewhat different character when the mast cells were exposed to *n*-decylamine. These findings confirm earlier results indicating that the release mechanism for compound 48/80 and bee venom is similar while there seems to be another mode of action for decylamine (Wilson 1954, Uvnäs 1957, 1960, Boréus 1960, Fredholm and Haegermark 1967). Differences between these liberators in their effects on mast cells were also noted in the electron microscope. These findings are in agreement with a previous investigation in which the ultrastructural changes and histamine release were studied after 10 min incubation with varying concentrations of these releasers (Bloom and Haegermark 1965, 1967). Moreover for a given level of histamine release there appears to be no difference between the morphological changes accompanying exposure to different liberator concentrations and those observed after interrupting the release reaction at some stage before being completed.

It could have been expected that the time required for the release process would somehow be related to the amount of histamine released. However it was found that for a given temperature the release curves of compound 48/80 and bee venom reached maximum levels after approximately the same time, independently of the amounts released. This would indicate that the reaction time of the cells is not determined by how much histamine is to be released.

It might also have been anticipated that enzyme inhibitors would influence the time required for the cells to release their histamine as the release mechanism for compound 48/80 and bee venom is known to involve cellular enzymatic processes. However when enzyme inhibitors were added in concentrations giving a partial inhibition of histamine release, the time curve had the same appearance as if a lower dose of liberator had been used. Thus, the final level of the curve was lowered, but the reaction time of the mast cells was not influenced.

At 22° C, using compound 48/80 or bee venom as liberators, there was a delay of 5—10 sec before any histamine was released. The significance of this lag phase is not possible to explain since at present we lack knowledge of the steps involved in the release process. It was observed that if we tried to terminate the release process by diluting the liberator ten fold, to a noneffective concentration, with salt solution of 22° C instead of 0° C as usual, the reaction was not stopped (Fig. 4 a). It continued

to completion even after 5 sec exposure to the adequate concentration of the liberator. This indicates that during the first 5 sec (perhaps still less) the release process had been initiated and that once started it could proceed even in the absence of an effective dose of releaser.

In another type of experiment the incubation was terminated after 5 sec in the usual way by cooling and diluting and then the tubes were re-warmed to 22° C. The results were somewhat contradictory to those in the previous section, as no release occurred. According to the previous findings release should have been initiated during the first 5 sec at 22° C in an appropriate concentration of 48/80. This might indicate that the releasing agent is reversibly bound to the cell receptors and is set free before the incubation temperature has returned to a level where the cell is able to react. Another explanation might be that somewhere in the sequence of reactions leading to histamine release, a short-lived intermediate product is formed. At 0° C the process is inhibited, and when a suitable temperature is reached again after 1—2 min, the postulated factor has disappeared. However these cells were able to respond if they were again exposed to an adequate concentration of compound 48/80 which means that their reactivity was not influenced by the short "activation" for 5 sec.

As the release curves obtained in this study represent the sum of events taking place in the whole cell population it is impossible to deduce from them what is happening in the individual cells. Thus, they cannot answer the important question whether increased histamine release is due to either the cells reacting with a successively increased release, or to more and more individual cells responding to the stimulation. The combined release studies and morphological observations seem to indicate that an increase in histamine release involves both these alternatives. It is unquestionable that the visible cellular changes increase with increasing histamine release, and roughly reach the same level in the majority of the mast cells in each phase of the release process. However it is also evident that differences in sensitivity towards liberators exist among the mast cells as there are always some cells which are more or less affected than the majority. The number of unaffected or slightly affected mast cells decreases with increased histamine release.

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Acta physiol. scand 1967 71 283—290

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The Effects of Compound 48/80 and Distilled Water on the Adenosine Triphosphate Content of Isolated Rat Mast Cells

By

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Received 7 June 1967

Abstract

Diamant B. *The effects of compound 48/80 and distilled water on the adenosine triphosphate content of isolated rat mast cells* Acta physiol scand. 1967 71 283—290

The ATP content of isolated peritoneal mast cells from 15 rats averaged 1.7 ± 0.1 mmoles/kg dry weight. When degranulation of the cells and histamine release was induced by compound 48/80 no significant decrease in the ATP content could be demonstrated. When buffered distilled water was used instead as degranulating and histamine releasing agent the ATP content of the cells decreased by 65—75 per cent with an apparent $t_{1/2}$ of about 20 sec. The results are discussed in relation to the postulated energy-dependent reaction involved in the degranulation by compound 48/80

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In following paper (Diamant and Krüger 1967) various observations were discussed which indicate that some stage in the mechanism of histamine release from isolated rat mast cells induced by compound 48/80 is energy-dependent. ATP generated by anaerobic glycolytic reactions seems sufficient to furnish energy for this process (Diamant 1960, Diamant and Uvnäs 1961) As pointed out by Uvnäs (1964, 196) the energy dependent step seems to be involved in those cellular reactions that cause the morphological changes in the cells (degranulation) since he could demonstrate that the actual release of histamine from isolated mast cell granules depends on a non-enzymic exchange of histamine in the granular matter for cations. Mast cell degranulation and histamine release always occur as parallel phenomena when the cells are exposed to compound 48/80 consequently if mast cell degranulation is blocked histamine release will not occur For the sake of convenience and quantitation, histamine release is frequently studied. Histamine release is considered to reflect morphological changes in rat mast cells. Thus, it is considered justifiable to draw conclusions regarding the mechanism by which compound 48/80 induces degranulation in mast cells by studying histamine release.

Little information is available concerning the energy-generating enzymic reactions that occur in rat mast cells. Schauer and Eder (1961) postulated a high content and turnover of ATP in rat mast cells, based on the histochemical demonstration of various phosphate-hydrolysing enzymes as well as on the observation by Korn (1959) that ATP is necessary for the incorporation of sulphate groups into the heparin molecule. Chakravarty (1965) demonstrated, using the Cartesian diver technique, that rat mast cells have a high glycolytic activity. Diamant (1967 b) pointed to the apparent absence of certain enzymes of the citric acid cycle and the hexose monophosphate shunt in rat mast cells. These data however do not give any quantitative information as to the content and utilization of ATP in rat mast cells.

In the present investigation ATP was determined in isolated rat peritoneal mast cells. The effects of compound 48/80 and buffered distilled water (hypotonic incubation medium) on the ATP content of the cells were studied. Both agents degranulate rat mast cells and cause the release of histamine compound 48/80 by the postulated energy-requiring mechanism and distilled water by a non-specific cell lysis. The ATP content of the mast cells was followed in order to see whether histamine release by an energy-dependent histamine releasing agent was reflected in a decrease of the ATP content of the cells.

Methods

Rat peritoneal mast cells were isolated and concentrated as described in a following paper (Diamant and Krüger 1967). 30 µl samples of the cell suspension were incubated in glass tubes (50 mm long, 6 mm outer and 4 mm inner diameter) at 37° C for various lengths of time as stated in the text. In experiments concerning the effect of compound 48/80 3 µl of solution was added, giving a final concentration of 2 µg/ml. When the effect of distilled water was investigated the tubes were centrifuged (350 × g for 10 min), the supernatant was discarded and 30 µl of distilled water buffered (pH 7.0) with 10 % v/v Sörensen phosphate buffer (67 mM) was added prior to the incubation at 37°. After incubation, 1.8 µl from each tube was transferred in duplicate (for histamine determination) to fluorometer tubes containing 2 ml of balanced salt solution with human serum albumin (0.1 %). To the remainder 3 µl of NaOH (0.2 N) was added and the tubes were heated for 10 min at 60° to stop enzyme activity in the cells. The tubes were then frozen and stored at −80° until ATP was determined (within 4 hrs). ATP standards and blanks were carried through the whole experiment and treated identically to the cell samples. Duplicate samples of cells, standards and blanks were used in all experiments.

The fluorometer tubes were centrifuged for 10 min at 350 × g, the supernatants were decanted and distilled water (2 ml) was added to the precipitates. Histamine was determined on supernatants and precipitates by the fluorometric method of Shore *et al.* (1959).

ATP was determined according to Lowry *et al.* (1964). Duplicate or triplicate determinations were made from each sample. 5 µl of the sample was mixed with 5 µl of Tris-HCl 0.1 M, pH 7.5 containing bovine serum albumin (0.01 %), $MgCl_2$ (5 mM), TPN⁺ (0.2 mM), glucose (1 mM), yeast hexokinase (1 µg/ml) and glucose-6-phosphate dehydrogenase (0.5 µg/ml). Prior to each experiment the amount of enzyme was adjusted so as to give a time for ATP of about 1.5–2 min. After 30 min at room temperature 20 µl of a solution containing Na_3PO_4 (0.3 M) and K_2HPO_4 (0.3 M) pH 11 was added and the samples were heated at 60° for 10 min to destroy excess TPN⁺. The whole sample was then transferred to and mixed vigorously with 1 ml of 6 N NaOH containing 0.01 % H_2O_2 and heated for 15 min at 60° according to Lowry *et al.* (1957). The fluorescence was read in a Farrand fluorometer model A 2.

The concentration of ATP in the mast cells was calculated as moles/kg dry weight, using the cell count (Bürker chamber) of the original cell suspension and the mean dry weight of rat peritoneal mast cells (Diamant and Lowry 1966).

Results

The ATP content of rat peritoneal mast cells isolated from 15 rats averaged 1 73 ± 0.10 mmoles/kg dry weight (mean ± S.E.) when tested immediately after isolation (Table I) Since the average dry weight of a rat peritoneal mast cell is about 500 pg, the ATP content would amount to about 8 7 × 10⁻¹ moles/cell. When mast cells from 3 rats had been incubated at 37° for 60 min 98 per cent (range 88—124 per cent) of the ATP content remained in the cells (Table I)

When mast cells from 4 rats were exposed to 48/80 (2 µg/ml) no difference in ATP content was noted between cells exposed for 5 and 60 min respectively While more than 50 per cent of the total histamine was released (not shown in Table I) the ATP content in both cases averaged 90 per cent (range 8?—99 per cent) of the values observed prior to incubation with compound 48 80.

When mast cells from 3 rats were incubated for 60 min at 37 with potato apyrase (5 µg/ml) no significant decrease in the intracellular ATP content was noted 97 per cent (range 96—100 per cent) of the content prior to the incubation being found. On the other hand when apyrase (5 µg/ml) was incubated with 12.4 × 10⁻⁸ M ATP (the concentration corresponding to what would be found if all the intracellular ATP of the mast cells at the time of incubation was present extracellularly) only 1—2 per cent remained after 60 min at 37°

In Fig 1 the ATP content of rat mast cells was determined after the cells had been exposed to compound 48 80 at 37 for 1/4 1/2, 1 2 and 5 min. No apparent change was noted and the values corresponded to the ATP content of control cells not exposed t compound 48/80 When the incubation time was extended (5 30 and 60 min, Fig 2) using mast cells from a second rat the ATP values at each incubation time were about 10 per cent less than those observed in the corresponding controls without compound 48 80 However the values of duplicate cell samples overlap. In the controls less than 10 per cent of the total histamine was released

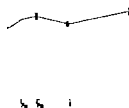


Fig 1 ATP content of ra mast cells af er incubation with compound 48/80 (2 γ/ml) 37 for 1/4 1/2 1 2 and 5 min. The results of duplicate cell samples are shown. Filled triangles denote ATP content of ells before the addition of compound 48/80 and after 5 min t 37 ithout compound 48/80

TABLE I

ATP content of rat mast cells (mmoles/kg dry weight)

Rat no.	Tested after isolation	Tested after incubation at 37°	Tested after incubation with compound 48/80 2 µg/ml at 37°
1	1.78		
2	1.39		
3	1.36		
4	2.08		1.71 (82%)
5	1.44		1.42 (99%)
6	2.05		1.89 (92%)
7	2.05		1.81 (88)
8	1.50	1.86 (120%)	
9	2.50	2.26 (90%)	
10	1.17	1.20 (103%)	
11	1.50	1.32 (88%)	
12	1.80		
13	2.45		
14	1.54		
15	1.90		
Mean ± S.E.	1.73 ± 0.10		

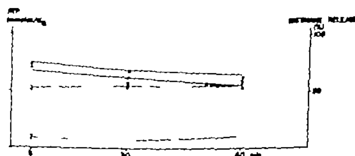


Fig. 2. Rat mast cells incubated with compound 48/80 (2 γ/ml) at 37° for 5, 30 and 60 min. Continuous lines: ATP content. Broken lines: histamine release. The results of duplicate cell samples are shown.
○ spontaneous histamine release from control cells without compound 48/80
● histamine release induced by compound 48/80 (spontaneous release deducted)
× ATP content of control cells without compound 48/80
■ ATP content of cells exposed to compound 48/80.

spontaneously. Compound 48/80 caused an additional 50 per cent release of histamine and the observed release was constant at all incubation times.

In Fig. 3 mast cells from a third rat were treated identically to those in the experiment shown in Fig. 1 but compound 48/80 was replaced by buffered distilled

Tested after incubation apyrase 5 μg/ml t 37°	Incubation time	ATP (M) Incubated with pyrase 5 μg/ml at 37 before	after
1 71 (96%)	60 min	12.4 × 10⁻⁴	0.17 × 10⁻⁴
1.39 (100%)	60 min	12 4 × 10⁻⁴	0.18 × 10⁻⁴
1.30 (96%)	60 min	12.4 × 10⁻⁴	<0.2 × 10⁻⁴
	60 min		
	60 min		
	5 min		
	5 min		
	60 min		
	60 min		
	5 min		
	60 min		

Per cent values denote ATP content in % of values observed prior to incubation



Fig 3. ATP content of ra mast cells incubated in buffered distilled water t 37 for 1/4 1/2, 1 2 and 5 min The results of duplicat cell samples are shown.

water During the first 5 min of incubation 74 per cent of the ATP of the cells disappeared. This disappearance took place exponentially with time with an pparent t for ATP of about 70 sec In Fig. 4 the cells from fourth rat were incubated for 5 30 ad 60 min with buffered dist lled water When compared with the ATP content of control cells that were incubated in buffered isotonic salt solution 65 per cent

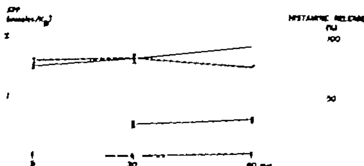


Fig. 4. Rat mast cells incubated in buffered distilled water and isotonic salt solution at 37° for 5, 30 and 60 min. Continuous line: ATP content. Broken lines: histamine release. The results of double duplicate cell samples are shown.
○ histamine release from cells incubated in isotonic salt solution
● histamine release from cells incubated in buffered distilled water
× ATP content of cells incubated in isotonic salt solution
■ ATP content of cells incubated in buffered distilled water

(range 63—66 per cent) of the ATP had disappeared. It is evident that after 5 min no further decrease in the ATP content of the cells incubated in distilled water occurred. The spontaneous histamine release in the controls was less than 5 per cent. An additional 75—84 per cent of the histamine (at the different times of incubation) was released from the mast cells after incubation with buffered distilled water.

Discussion

In some, but not all, experiments in the present investigation a slight decrease in the intracellular ATP of rat mast cells was noted when histamine release was induced by compound 48/80. This effect, however, was not statistically significant. Several possible reasons for this negative finding have to be excluded before it can be considered justified to draw the conclusion that ATP is not needed and utilized in the degranulation mechanism induced by compound 48/80. Firstly, it should be noticed that even though no ATP-generating substrates (e.g. glucose) were added to the cells either during the isolation procedure or during the actual incubation of the cells, the ATP content of cells from different rats was remarkably constant and did not change significantly during incubation at 37° for 60 min. This indicates that the turnover of ATP in the cells is low and/or that endogenous substrates are present intracellularly in sufficient amounts and are utilized to maintain a constant ATP level. No quantitative information is yet available as to the intracellular concentrations of glucose, glycogen and creatine phosphate and whether or not these substrates decrease with prolonged incubation or after the exposure of the cells to compound 48/80. Secondly, it is possible that the amounts of ATP that are utilized by the degranulating mechanism are so small that it is not possible to register the changes with the experimental procedures used. From the present data the intra-

cellular concentration of ATP in rat mast cells can be calculated to amount to about $3.5 \times 10^{-}$ M if it is assumed that it is evenly distributed within the cell. It is also possible that the same concentration of ATP that is effective in inducing histamine release when present extracellularly (Diamant and Krüger 1967) might also be effective when present in the cell membrane. In that case a concentration of only 5—10 per cent of the calculated intracellular concentration would be enough to furnish energy for the reactions involved in the degranulation induced by compound 48/80. This might also help to explain the negative findings regarding ATP utilization in the present investigation.

On the other hand, when rat mast cells were degranulated by cell lysis using buffered distilled water the ATP content quickly decreased by 65—75 per cent. This can be explained by the recent demonstration of an ATP-hydrolysing enzyme, located in the cell membrane with its effective sites presumably directed outwards from the cell (Diamant 1967 a). Evidently after degranulation of the cells induced by distilled water the intracellular ATP quickly reaches this ATP hydrolysing enzyme. The finding that 25—35 per cent of the ATP remained unchanged indicates that this part might be bound to cell structures in such a way that it never becomes accessible to the enzyme.

For obvious reasons, degranulation induced by compound 48/80 must be far less deleterious to the integrity of the cell structure since the endogenous ATP never seems to reach the ATP-hydrolysing enzyme. In this respect similar results were reported (Diamant 1967 b) concerning lactic dehydrogenase of rat mast cells. This enzyme did not leak out from the cells after they were exposed to compound 48/80 whereas this was the case when they were incubated in a hypotonic solution.

It has been postulated that the function of ATP could be to bind histamine in mast cells (Sanyal and West 1956, Eder and Schauer 1961). When the quantitative data for the intracellular ATP in the present investigation (8.7×10^{-8} moles/cell) is compared with the average histamine content of a mast cell (20 pg as histamine base or 1.8×10^{-14} moles/cell) the molar ratio of ATP to histamine amounts to 1:200. This ratio is similar to that observed by Johansson and Uvnäs (unpublished observations, see Uvnäs and Thon 1966) and further supports the opinion that ATP cannot be of significant importance for the storage mechanism of histamine in mast cell granules of the rat.

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Histamine Release from Isolated Rat Peritoneal Mast Cells Induced by Adenosine-5 Triphosphate

By

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Abstract

Diamant B. and P G Krüger, *Histamine release from isolated rat mast cells by adenosine-5'-triphosphate* Acta physiol. scand 1967 *71* 291—302.

Histamine release from isolated rat mast cells is induced by ATP ($10^{-[illegible]}$ — $3 \times 10^{-[illegible]}$ M) The activity found in an ADP preparation was shown to be due to contamination with ATP purified ADP has no activity AMP 3 5 AMP PCr and PEP were without histamine releasing activity Histamine release induced by ATP was compared with that caused by compound 48/80 with respect to the influence of Ca^{++} Mg^{++} Zn^{++} ouabain, DNP oligomycin and glucose. The results are interpreted to indicate that ATP and compound 48/80 release histamine by mechanisms that in certain respects differ from each other The histamine releasing effect of ATP is discussed in relation to the energy-requiring mechanism that is known to be involved in histamine release by compound 48/80.

The reactions leading to histamine release from tissues and from isolated mast cells are generally considered to be in some part energy-requiring So far however all the evidence available to support this theory is circumstantial. The original observation favouring this hypothesis was mad by Parrot (1942) who showed that antigen-induced histamine release did not occur from isolated sensitized guinea-pig lung tissue in the absence of oxygen. Similar observations have since been made using various *in vitro* preparations and a variety of histamine releasing agents. These have been put forward as evidence for the dependence of histamine release on an intact oxidative phosphorylation (Mongar and Schild 1957 Moussatché and Provoust Danon 1958, Chakravarty 1959) However the release of histamine was found to occur even under nitrogen a long as the tissues or mast cells were preincubated with glucose, pointing towards the possibility that glycolytic generation of adenosine triphosphate (ATP) would suffice to furnish energy for some so far unknown energy requiring step in the reactions leading to histamine release (Diamant 1960 Diamant and Uvnäs 1961) This effect of glucose is counteracted by phlorizin, known to

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inhibit the transport of glucose through cell membranes (Diamant 1962). In addition, glucose also reduces the inhibitory effect of certain metabolic inhibitors (2,4-dinitrophenol, cyanide, azide) on histamine release (Diamant and Uvnäs 1961; Rothschild, Vugman and Rocha e Silva 1961; Moran, Uvnäs and Westerholm 1962). In this respect histamine release induced by antigen-antibody reactions, compound 48/80, extracts of *Ascaris suis* and *Cyanea capillata*, bee venom and chymotrypsin seem to be equally dependent on energy being available at the time of the induction of histamine release from tissues as well as from isolated rat mast cells.

From recent observations by Lagunoff and Benditt (1963) and Uvnäs (1964) it is evident that mast cell granules retain their histamine when they are isolated in an ion-free medium and that on the addition of small amounts of cations these will rapidly replace histamine and cause its release. This process is influenced neither by temperature nor by metabolic inhibitors and can be reversed by the addition of sufficient amounts of histamine whereby the cations already exchanged for histamine will be replaced. These observations indicate the passive, non-enzymatic, ion-exchange-like nature of the process. The physiological cation concentration found in the extracellular fluid is such that all the histamine in mast cell granules will rapidly be exchanged for cations on meeting such a medium (Uvnäs 1964).

Since histamine release from isolated rat mast cells induced by the above-mentioned agents is known to be accompanied by morphological changes (degranulation) the granules do indeed meet such a cation-containing medium. This degranulation process, on the other hand, is dependent on physiological temperature and pH and can be completely inhibited by anoxia or metabolic inhibitors, the latter effects being fully counteracted by the presence of glucose (Högberg and Uvnäs 1957; Diamant and Uvnäs 1961). Consequently, due to the inhibition of the degranulation process histamine release will not occur. Therefore, the morphological changes in mast cells can be considered to be the initial and active part of the cellular reaction and histamine release from the granules as a secondary and passive phenomenon (Uvnäs 1966, 1967).

Rat mast cells contain about 1.7 mmoles/kg dry weight of ATP (Diamant 1967a). If it is assumed that the cell contains about 80 water this would correspond to a concentration of about 5.5 10 M if the ATP was evenly distributed within the cell. Recently, Keller (1966) found that when mast cells were incubated with ATP (10 M) or ADP (5 10 M) the cells were damaged and more than 75 of the histamine was released. This effect was ascribed to a direct cytotoxic action.

In view of the observed intracellular concentration of ATP and the indirect evidence for the involvement of ATP in the reactions leading to histamine release the ability of some organic phosphate esters to release histamine from isolated rat mast cells was investigated. This was also done to determine whether more "physiological concentrations" of the compounds would be effective. In addition the histamine releasing activity of ATP was compared with that of compound 48/80. The effects of temperature, Ca , Mg , Zn , ouabain, oligomycin, DNP as well as the influence of glucose on the release by these two agents were investigated.

Methods and materials

Isolation of rat peritoneal mast cells

Peritoneal mast cells were isolated from Sprague-Dawley rats (males, weight 350—400 g) *ad modum* Uvnäs and Thon (1959). After separation of the mast cells in the Ficoll gradient the cells were washed 3 times each time with 3 ml of a balanced salt solution containing NaCl (154 mM), KCl (2.7 mM) and $CaCl_2$ (0.9 mM) buffered (pH 7.0) with 10 % v/v Sörensen phosphate buffer (67 mM). In addition, the solution contained human serum albumin (0.1 %) (kindly supplied by AB Kabi, Sweden). The cells were finally suspended in 150 µl of this solution and counted in Bürker chamber; the cell concentration varied with different rats between 2.5—4 × 10^4 per ml. In experiments concerning the effect of Ca^{++} this ion was omitted from the balanced salt solution in all steps of the isolation procedure.

Incubation procedure

Two ml of the balanced salt solution was added to matched fluorometric tubes suitable for reading in a Farrand fluorometer Model A 2 (Farrand Optical Co. Ltd. USA). After the addition of the various phosphate esters or compound 48/80 (kindly supplied by AB Leo, Sweden) the tubes were preincubated for 5 min at 37° C. 22 µl of the mast cell suspension was then added. After 10 min at 37° the tubes were centrifuged for 10 min at 350 × *g* and the supernatants were decanted into new fluorometer tubes. 2 ml of distilled water was added to the residue to release all the remaining histamine from the cells.

In experiments concerning the influence of inhibitors and cations the cells were preincubated with these agents for 10 min at 37°. The reaction was then started by the addition of ATP or compound 48/80. In experiments investigating the effect of glucose an additional preincubation period with glucose (5.6 mM) for 15 min at 37° preceded the addition of the inhibitor. Further details regarding the experimental procedures are given in the text.

All samples were run in duplicate. The histamine release is expressed as percentage of the total histamine content of the sample and the mean value of the duplicates used. The spontaneous histamine release was deducted from all values unless otherwise stated.

Histamine determination

The histamine in the supernatants and the corresponding residues was determined by the fluorescence method of Shore et al (1959). The extraction procedure of this method was omitted, however in accordance with the findings of Bergendorff (1965) that histamine is the only substance present in rat mast cells that gives a registrable fluorescence with o-phthalaldehyde.

The fluorescence was read in a Farrand fluorometer Model A 2 using a primary glass filter Corning 5840 and secondary filters 3387 and 4303 in combination. In order to keep the temperature of the fluorometer constant when the mercury arc lamp was used a more powerful fan (Pabst Motoren, Germany type 2503) was fitted to the back of the fluorometer. For greater convenience and accuracy a microammeter (Norma, Austria, model 621 accuracy 0.5 %) was connected to the amplifier of the microammeter unit supplied with the fluorometer.

Materials

Phosphate esters (as sodium salts) were obtained from Sigma Corporation, USA. Ouabain was kindly supplied by Sandoz Ltd. Switzerland. Stock solutions (about 100 mM) of ATP, ADP, AMP and PEP were assayed spectrophotometrically by enzymatic methods used in the laboratory of Dr. O. H. Lowry, St. Louis, Mo. USA. The stock solutions of PCr and 3'5'AMP were prepared by weighing out the substances (taking into account the degree of purity quoted by Sigma). When necessary (ATP and ADP) the stock solutions were adjusted to pH 7.0 by the use of N OH. All stock solutions were stored at —20°, which in the cases of ATP and ADP did not influence the activity over a period of at least 1 month.

Oligomycin (Sigma) was dissolved in ethanol (95 %). In experiments with oligomycin equal amounts of ethanol were added to controls without oligomycin to ensure that the ethanol did not influence the histamine release.

Abbreviations

ATP	Adenosine-5'-Triphosphate
ADP	Adenosine-5'-Diphosphate
AMP	Adenosine-5'-Monophosphate
PCr	Phosphocreatine
PEP	2-Phosphoenolpyruvic Acid
3'5'AMP	Adenosine-3'5'-Cyclic Monophosphate
DNP	2,4-Dinitrophenol

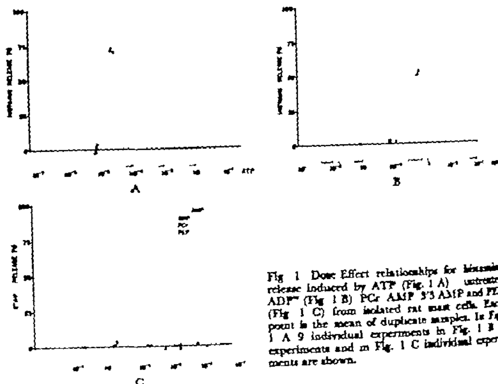


Fig 1 Dose Effect relationships for histamine release induced by ATP (Fig. 1 A) untreated ADP (Fig 1 B) PCr AMP 3'5 AMP and PEP (Fig 1 C) from isolated rat mast cells. Each point is the mean of duplicate samples. In Fig. 1 A 9 individual experiments in Fig. 1 B 4 experiments and in Fig. 1 C individual experiments are shown.

Results

Isolated rat mast cells released their histamine when exposed to ATP (Fig 1 A) and ADP (Fig 1 B) They were not affected by AMP 3 5 AMP PEP or PCr over the range of concentrations tested (in most cases between 2×10^{-4} M and 10^{-3} M) (Fig. 1 C) In addition adenosine was also ineffective in the same concentrations (Dahlqvist and Diamant, unpublished observations)

For ATP the dose-effect relationship varied somewhat with different rats as judged from 9 experiments presented in Fig 1 A. Histamine release was detectable when the cells were exposed to a concentration of approx. 10 M ATP and optimal release (70—80) was obtained with about 3×10^{-4} M ADP was less effective (Fig 1 B). No histamine release was observed below 5×10 M and optimal release (70—80 per cent) was elicited with about 3×10 M ADP

When the concentrations of ATP and ADP required to induce 50 per cent of the optimal histamine release were compared (Fig 1 A and B) it was evident that the histamine release induced by ADP could be accounted for if the commercial ADP preparation was contaminated with 2.5—3 of ATP For this reason two stock solutions of ADP were tested by enzymatic methods spectrophotometrically for ATP and it was found that they were contaminated with 1.8 % and 2.1 % ATP respectively (molar relationships) In order to test this explanation further the experiment shown in Table I was performed. When mast cells were exposed to 1.33×10^{-4} M untreated ADP 73 per cent of the histamine was released. When, however prior

TABLE I Pretreatment of stock solution of ADP (75.6 mM)

I	II	III	IV
9 ml ATP-reagent[1]	9 ml ATP-reagent	9 ml ATP-reagent	9 ml ATP-reagent
162 μl ADP (75.6 mM)	162 μl ATP-reagent	162 μl ADP (75.6 mM)	162 μl ATP-reagent
20 μl enzyme mixture	20 μl enzyme mixture	—	—

Incubation at room temperature for 30 min. Enzymes heat killed at 100° for 2 min.

Histamine release from isolated rat mast cells incubated in solutions treated as above.

I	II	III	IV
5.6 %	4.0 %	73.4 %	1.2

Final concentration of ADP for histamine release: 1.33 × 10^{-3} M. By spectrophotometric determination the ATP-contamination amounted to 2.8 × 10^{-5} M or 2.1 %. Spontaneous histamine release not deducted.

ATP-reagent Balanced salt solution (pH 7.0) containing human serum albumin (0.1 %) $MgCl_2$ (2 × 10^{-3} M) glucose (10^{-3} M) and TPN (3 × 10^{-4} M).

Enzyme mixture Glucose-6-phosphate Dehydrogenase (5 mg/ml) and Hexokinase (10 mg/ml) (Boehringer and Son, Germany) giving final dilution of 1 7700 and 1 5000 respectively after the addition to the reagent.

to the addition to the cells the ADP was treated with a reagent that converted ATP to ADP (see Table I) only 6 per cent of the histamine was released from the cells. It was therefore concluded that ADP is ineffective as a histamine releasing agent and that the observed releasing activity was due to contamination with ATP

It was of interest to investigate if the addition to the cell suspension of a system competing for ATP would influence the histamine release induced by ATP (Fig 2) Potato apyrase (Sigma) was used as the competing system. Apyrase (2.5 or 25 μg/ml) itself had no histamine releasing effect. Furthermore, it did not activate the histamine release when ATP was used at a concentration (0.8 × 10^{-4} M) that was

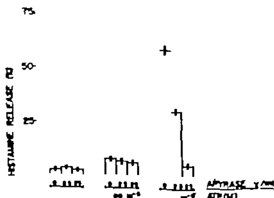


Fig. 2. The effect of potato apyrase on the histamine release from isolated rat mast cells induced by ATP Individual values of duplicate samples are shown. Incubation procedure: balanced salt solution containing human serum albumin, apyrase mast cells and ATP was added in this order to tubes kept on ice. These were then incubated for 10 min 37° C.

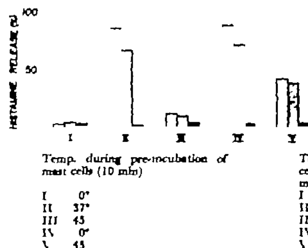


Fig. 3. The influence of temperature on histamine release from isolated rat mast cells induced by ATP (hatched bars) and compound 48/80 (open bars). Spontaneous histamine release (filled bars) not deducted. Incubation procedures

Temp. during pre-incubation of mast cells (10 min)

I	0°
II	37°
III	45
IV	0°
V	45

Temp. during incubation of mast cells with histamine releaser (10 min)

I	0°
II	37°
III	45
IV	37°
V	37°

Care was taken to let the tubes adjust to th ew temperature before the addition of the histamine releaser. This was ensured by incubating the tubes for 30 sec t the new temperature. ATP and compound 48/80 sol tions were preheated to the temperatures of the cell suspension.

slightly below the minimal effective concentration. On the contrary histamine release by ATP was inhibited by apyrase. 57 per cent of the histamine content was released by 2 9 × 10 M ATP but in the presence of 2.5 µg/ml of apyrase the histamine release decreased to 29 5 per cent and with 25 µg/ml of apyrase to 5 per cent. These findings indicate that apyrase competes with the cells for ATP and that hydrolysis of ATP by apyrase in the incubation medium does not trigger histamine release. In add tion the initiating agent for histamine release by ATP cannot be a product formed by the extracellular hydrolysis of ATP by apyrase

In view of earlier indications that ATP might play a significant role in histamine release induced by other agents the releasing effect of ATP was compared with that of compound 48/80 in regard to the effects of temperature, various cations and inhibitors. In the experiments described below ATP and compound 48/80 were used in concentrations of 3 10 M and 2 µg/ml respectively

Histamine elease by both agents was equally sensitive to temperature changes (Fig 3) At 0 and 45 histamine release was inhibited. Pretreatment of the cells at 0° for 10 min prior to exposure to ATP or compound 48/80 at 37° did not affect the release as compared with the release from cells preincubated at 37° indicating the reversible nature of the inhibition at 0 Pretreatment of the cells at 45 for 10 min prior to exposure to the releasers at 37 caused a 50 per cent inhibition of the histamine release indicating a destruction of some part of the mechanism leading to the release of histamine In this respect the present results differ from those of others (Uvnäs and Thon 1961 Saeki 1964) who found that pretreatment at 45 completely inhibited subsequent release by 48/80 at 37° C.

When histamine release was induced in the absence of Ca^{++} the release by compound 48/80 decreased to 75—85 per cent of the values observed when the cells were

TABLE II. The influence of Ca^{++} Mg^{++} and Zn^{++} on histamine release from rat mast cells by ATP and compound 48/80.

Exp.	Cation	Histamine releaser	Cation (M)					
			0	10^{-}[illegible]	10^{-}[illegible]	10^{-}[illegible]	10^{-}[illegible]	10^{-}[illegible]
1	Ca^{++}	48/80	61	56	63	62	63	84
		ATP	6	5	5	13	43	72
2	Ca^{++}	48/80	64		59	59	60	76
		ATP	5		16	7	31	70
3	Mg^{++} (Ca^{++} absent)	48/80	56	53	54	5	52	43
		ATP	4	2	3	3	7	3
4	Mg^{++} (Ca^{++} 10^{-}[illegible] M present)	48/80	72	73	72	73	72	71
		ATP	71	68	61	63	59	23
5	Zn (Ca^{++} 10^{-}[illegible] M present)	48/80	72		72	74	69	43
		ATP	58		54	48	9	2

All values denote histamine release as percentage of the total histamine content of the cells. Means of duplicates.

preincubated with Ca^{++} (10^{-}[illegible] M) for 10 min prior to the addition of compound 48/80 (Table II exp. 1 and 2). ATP-induced histamine release was found to be much more sensitive to the lack of Ca^{++}. The corresponding values amounted to less than 10 per cent and a graded inhibition was observed with decreasing concentrations of Ca^{++} (Table II exp 1 and 2). Mg^{+} could not be substituted for Ca^{++} in the reactions (Table II exp 3). In fact in the presence of Ca^{++} (10 M) Mg^{++} (10^{-} M) caused an inhibition (66 per cent) of the histamine release induced by ATP whereas the release by compound 48/80 was not affected (Tabl II exp 4).

In the presence of Ca^{++} (10 M) Zn (10 M) caused an inhibition of compound 48/80-induced histamine release that amounted to 38 per cent, but it was ineffective at 10 M. Histamine release induced by ATP was far more sensitive to Zn^{++}. At 10 M 97 per cent and, at 10 M 83 per cent inhibition was observed (Table II exp 5).

In all experiments (Tabl II) qualitatively the same results were obtained when instead of preincubating the cells with the cations these were added to th cells together with the histamine releasing agent (Diamant and Krüger unpublished observations).

Histamine release induced by ATP was found to be slightly mor sensitive to DNP than that caused by compund 48/80. Whereas 10 M DNP (in th absence of glucose) did not affect histamine release by compound 48/80 about 50 per cent inhibition was found when ATP was used as the releasing agent (Table III exp. 1 and 2). Higher concentrations complet ly blocked histamine release by both agents. From

TA LE III The influence of DNP nd glucose on histamine release from rat mast cells by ATP nd compound 48/80.

Exp.	Glucose (5.6 mM)	Histamine releaser	DNP 0	5×10^{-4}	10^{-4}	5×10^{-4}	10^{-4}	10^{-4}
1	no	48/80	76		77		4	0
	no	ATP	70		57		1	0
2	no	48/80	68				5	
	yes	48/80	67				57	
	no	ATP	67		39		3	
	yes	ATP	70		73		33	
3	no	48/80	57			11		
	yes	48/80	58			60		
	no	ATP	57	49		2		
	yes	ATP	63	63		34		

All values denote histamine release as percentage of the total histamine content of the cells. Mean of duplicates.

T BLE IV The influence of ouabain on histamine release from rat mast cells by ATP and compound 48/80

Glucose (5 6 mM)	Histamine releaser	Ouabain (M) 0	10^{-4}	10^{-}	10^{-4}	10^{-4}	10^{-4}	10^{-4}
no	ATP	78	77	78	77	77	77	78
yes	ATP	84	81	86	82	85	82	81
no	48/80	76	68	75	75	69	69	73
yes	48/80	79	73	79	78	79	74	76

All values denote histamine release as percentage of the total histamine content of the cells. Mean of duplicates

exp. 2 and 3 Table III it is also evident that glucose (5 6 mM) counteracted the inhibition by DNP of the release caused by compound 48/80 as well as that caused by ATP

Ouabain (Table IV) did not influence histamine release by ATP or compound 48/80 either in the presence or absence of glucose.

Oligomycin (Table V) wa found to be a remarkably effective inhibitor of histamine release by both agents. For reasons as yet unknown, the minimum effective concentration varied in different experiments between 10 and 10^{-4} M in the absence of glucose (Table V exp. 1—4) Both releasing agents seemed to be about

TABLE V The influence of oligomycin and glucose on histamine release from rat mast cells by ATP and compound 48/80.

Exp.	Glucose (5.6 mM)	Histamine releaser	Oligomycin (M) 0	10^{-14}	10^{-12}	10^{-10}	10^{-8}	10^{-6}	10^{-5}	10^{-4}	10^{-3}
1	no	ATP	72				2	0	1		
	no	48/80	81				14	0	0		
2	no	ATP	71			0	1	3			
3	no	ATP	60		66	59	63	0			
	yes	ATP	67		75	68	72	12			
4	no	ATP	68	69	68	65	2				
	yes	ATP	74	73	74	74	27				
5	no	48/80	64					2	1	2	2
	yes	48/80	72					68	65	65	67
6	no	48/80	68						1		
	yes	48/80	71						73		
	no	ATP	66						0		
	yes	ATP	78						22		
7	no	48/80	59				65	29			
	yes	48/80	66				69	65			
	no	ATP	67				59	4			
	yes	ATP	75				76	19			

All values denote histamine release as percentage of the total histamine content of the cells. Mean of duplicates.

equally sensitive to the action of oligomycin in any given experiment (Table V exp 1 6 and 7) W th respect to the counteracting effect of glucose there was a definit difference between the two agents investigated. In the presence of glucose oligomycin, even in concentrations of 10^{-3} M, did not inhibit histamine release elicited by compound 48/80 (exp. 5) whereas glucose caused only a slight reversal of the inhibition by oligomycin when histamine release was induced by ATP (exp 3 and 4) In exp. 6 and 7 where compound 48/80 and ATP were used in the same experiment, this d fference is further demonstrated. Therefore, it is concluded that glucose counteracts oligomycin-inhibition of histamine release when induced by compound 48/80 whereas this effect of glucose is much less pronounced in the case of release by ATP

D scussion

The observation that ATP induced histamine release from isolated rat ma t cells ga e rise to th hypothesis that the activation of intracellular ATP might be the common triggering factor in histamine release by those agents that are known to

induce mast cell degranulation by an energy-requiring mechanism. An activation of ATP could come about if these agents primarily influenced the intracellular distribution, formation or hydrolysis of endogenous ATP thus causing it to accumulate above a certain minimal effective concentration at some ATP-sensitive structure in the cell membrane. Considering the high intracellular concentration of ATP (Diamant 1967 a) as compared to the minimal effective concentration of ATP causing histamine release this seemed to be a reasonable possibility. In addition, histamine release induced by compound 48/80 or ATP was found to be about equally sensitive to oligomycin (in the absence of glucose), DNP and changes of temperature.

Although such a mechanism has not been disproved by the present investigation, some important differences exist between histamine release induced by ATP and compound 48/80 which do not seem to fit such a hypothesis. Firstly, histamine release by ATP was found to be dependent on Ca^{++} and was inhibited by Mg^{++} whereas compound 48/80-induced release was much less sensitive to the influence of these ions. The same insensitivity towards these ions was reported earlier by U[illegible] and Thon (1961) and Saeki (1964) in regard to histamine release induced by compound 48/80 from isolated rat mast cells. The effects of these ions on histamine release by ATP could be taken to indicate the involvement of a cellular ATP hydrolysing enzyme which would not necessarily be of importance in the release mechanism induced by 48/80. It was recently demonstrated that intact rat mast cells hydrolyse ATP added to the incubation medium, indicating that such an enzyme is present in the cell membrane (Diamant 1967 b). Secondly, glucose completely reversed the inhibition by oligomycin of histamine release caused by compound 48/80 whereas glucose was less effective in case of histamine release by ATP.

These findings do not fit in with the idea that compound 48/80 would react with the mast cells inducing a local accumulation of ATP. Instead it seems more probable that we are dealing with two different mechanisms of histamine release.

It has recently become apparent (Diamant and Krüger 1967) that ATP and compound 48/80 act on the mast cells by mechanisms that, at least in certain aspects, must be different. The morphological changes that are observed in mast cells after exposure to ATP differ distinctly from those induced by compound 48/80. The typical "degranulation" seen after compound 48/80 was never observed in cells treated with ATP and in addition, the morphological changes that did occur were reversible upon prolonged incubation of the cells. The interpretation of these findings will have to wait until further experiments are performed to correlate the morphological changes observed after ATP with the histamine release induced.

It is difficult to explain why the inhibition caused by DNP of histamine release induced by ATP is reversed by the presence of glucose. This seems to indicate, however, that intracellular ATP derived from the metabolism of glucose may play a role in the mechanism of histamine release by ATP just as it seems to do for compound 48/80 under similar conditions.

The high sensitivity of the histamine release by both agents towards oligomycin

is interesting in view of the effects on energy metabolism and on ATP-dependent changes in membrane structures reported for this substance. Oligomycin inhibits ATP-ase activity in mitochondria and submitochondrial particles (Lardy *et al.* 1958, Van Groningen and Slater 1963). A DNP-oligomycin-sensitive ADP-ATP exchange reaction was demonstrated in intact mitochondria (Wadkins and Lehninger 1963). Oligomycin inhibits mitochondrial swelling as well as ATP-induced contraction (Chapelle and Grevill 1961, Lehninger and Neubert 1962). Thus the sensitivity of histamine release to oligomycin indicates different possibilities for the inhibition of ATP-dependent cellular functions.

It is also important to consider the work of Nakao *et al.* (1960, 1961) who found that erythrocyte ghosts changed their shape in the presence of ATP. Such ghosts also contain an ATP-ase, the activity of which seems to be linked to the morphological changes of the cells. In the present investigation histamine release was found to be specifically induced by ATP. ADP and AMP were not effective. In this respect the results run paralell to the observations on the contraction phenomenon of swollen mitochondria which is also specifically induced by ATP whereas ADP and AMP are usually ineffective (Lehninger 1962).

The mechanism of histamine release by extracellular ATP and the apparent dependence of various histamine releasing agents on intracellular ATP might reflect the involvement of different ATP-dependent mechanisms. Although much has been speculated regarding the triggering of the cellular reactions leading to the release of histamine, nothing definite is known as yet. Therefore the activation of transport systems, contraction of cellular membranes or some other ATP-dependent chemical reaction are likely hypotheses, equally justifiable at the present state of knowledge. The effect of extracellular ATP on mast cells constitutes, however, an interesting area for future investigation since it deals with the action of a physiologically occurring energy-rich compound on a cellular membrane, causing morphological changes in the cell and the release of a biologically active amine.

Whether histamine release by ATP is of physiological importance deserves investigation. It is known (Duff *et al.* 1954) that ATP injected into the brachial artery of man acts as a potent vasodilator. The mechanism of this effect has not so far been elucidated but as judged from the present results it could be due to the release of histamine from mast cells.

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Degranulation and Histamine Release, Two Consecutive Steps in the Response of Rat Mast Cells to Compound 48/80

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Abstract

THON, I. L. and B. UVNÄS. *Degranulation a d histamine release two consec tive steps in the esponse of rat mast c lls to compound 48/80* Acta physiol. scand. 1967 *71* 303—315

Isolated rat peritoneal mast cells were exposed to compound 48/80 in *A* isotonic salt solution and in *B* isotonic sucrose solution. I both cases degranulation and histamine release occurred. In *A* all the released histamine ppeared dissolved in the suspension medium, the discharged granules having lost their histamine. In *B* the discharged granules were shown to retain histamine These granules released their histamine when suspended in NaCl-containing medium. Depleted granules could be refilled with histamine by suspension in histamine-containing medium and again depleted of the amine by exposure to sodium chloride. The proposal is made that the histamine release induced by compound 48/80 is two stage process, primary energy-requiring transport of histamine-containing granules t the outside of the mast cell and secondary nonenergy-requiring physico-chemical process, an extracellular cation exchange in the shed granules between histamine and cations, mainly sodium in the tissue fluid.

Rat mast cells respond to histamine releasing agents with extrusion of granules (degranulation) and release of histamine When the releasing agents are polymers such as antigens (to sensitized cells) (Hogberg and U nås 1960 Mota and Ishi 1960 U nas 1962, 1964) polypeptides from *As rs Cyanea* and other lower animals (U nas *et al.* 1960 U nas 1960) or synthetic polyamines *e.g.* compound 48/80 etc (Diamant and U nas 1961 U nås 1963) the mast cell response is in one way or another dependent on metabolic energy in the cell. It is blocked or reduced by inhibition of oxidative (in the bsence of glucose) as well as of glycolytic (in the absence of oxygen) metabolism (for references see Uvnås 1963)

The morphobological and biochemical events (the degranulation and the release) initiated by polymer agents are likely to be intimately related, the mast cell response being dependent on the same stimulatory nd inhibitory factors e.g. ionic milieu, pH, temperature enzyme inhibitors etc. (U nås and Thon 1961)

In spite of the pparent time-relationship between degranulation and release the

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abdominal fluid was sucked off with drop pipette. I similar way the pleural cavities were flushed with saline (4.5 ml) through an opening made in the diaphragm just below the xiphoid process.

Mast cells were isolated by differential centrifugation in Ficoll as described previously (Uvnäs and Thon 1961) with the minor modification that only two layers of Ficoll (30 and 40 per cent by weight) were used. Before experiments were performed in sucrose the mast cells were washed once in isotonic sucrose solution t 0 C, following the regular washings in isotonic salt solution. Care was taken not to prolong unnecessarily the exposure of the mast cells to sucrose, since the cells tended to shrink and to lose granules and histamine. Because of the tendency of both intact mast cells and mast cell fragments to adhere to glass, especially when suspended in ion-free media (distilled water or sucrose solution) as many procedures as possible were performed in plastic tubes (IEC No 2810).

Isolation of mast cell granule

When mast cells were degranulated by compound 48/80 in isotonic salt solution, pH around 7 the discharged granules showed great tendency to adhere to the surface of their mother cells by few granules appearing in the suspension medium. At pH 8 the adhesion was weaker but still several washings at this pH were needed in order t remove most of the extruded granules. The yield of granules obtained by differential centrifugation was still rather low however since the "free granules had great tendency to adhere to each other forming heavy clusters sedimenting together with the mast cells.

When mast cells were degranulated by compound 48/80 in isotonic sucrose pH 7 the discharged granules showed less tendency to adhere to their mother cells and to each other. A rich harvest of granules could be obtained by differential centrif gation at 2 700 × *g* of the supernatant after the initial run at 90—370 × *g*

Histamine as tested on atropinized guinea-pig ileum in Tyrode solution with continuous aeration at 37 C. All values were expressed as histamine base

T empty the sediments of their histamine the material was boiled for 5 min in isotonic saline. Control samples obtained by boiling in 0.01 N HCl showed the procedure to be 100 per cent effective

F all mol wt. 370 000 Pharmacia Co, Uppsala, Sweden) was dissolved in 0.9 per cent sodium chloride solution buffered to pH 7 with 10 per cent Sorensen phosphate buffer (2 hrs at 60 C) Dissolved human serum albumin was added to 0 1 per cent.

Isotonic sucrose 0 28 M

Isotonic sucrose pH 6.9 Isotonic sucrose was adjusted with KOH or N OH to pH 6.9 (final conc 10^{-4} M)

Isotonic salt solution pH 6.9 1.5×10^{-1} M NaCl, 2.7×10^{-3} M KCl 10^{-3} M $CaCl_2$ 10 per cent Sorensen phosphate buffer 0 1 per cent human serum albumin.

Results

Re ponse f mast c ll t peated e posure to compound 48/80

A. Mast cells suspended in isotonic salt sol tion

When suspended under favourable conditions rat mast cells react promptly and vigorously on exposure to compound 48/80 w th the extrusion of granules from the cells. However only few of these granules pass into the suspension medium, most of them sticking to the surface of the mother cell giving t mulberrylike appearance (Fig. 3A) In most of the cells, which ha now lost their normal semi-transparent appearance, no cell membrane is visible under the layer of shed granules. In some of the cells a more or less rugged" membrane can be imagined inside the layer of granules, but t is impossible to decide whether the "ruggedness of this membrane is an optical illusion due to changed refraction in the extracellular granules or is due to damage caused by the degranulation. Such a mulberry" does not respond to new dose of compound 48/80 The nomenclature used by



Fig. 3A. Degranulation responses of isolated mast cells to two consecutive exposures to compound 48/80 (1 μg/ml), pH 6.9, 37° C. Magnific. × 660.
1) Control cells (upper left).
2) Cells after 1st exposure to compound 48/80 (upper right).
Note: The adhesion of extruded granules to the cells.
3) Degranulated cells washed three times at pH 7.8, 37° C) (lower left).
Note: The absence of extruded granules and the rather normal appearance of the cells.
4) Cells after 2nd exposure to compound 48/80 (lower right).
Note again the adhering extruded granules after second degranulation.

longer with compound 48/80 the greater the second response of the cells. After 4 washings the cells released 39 per cent of their remaining histamine content.
The recovery of the responsiveness of the mast cells to compound 48/80 depended not only on the number of washings but also on the pH of the wash fluid (compare in Fig. 2 the difference between the effects of washing at pH 5.7 and 8.0).
The morphological appearance of normal, degranulated, washed and redegranulated cells is shown in Fig. 3A. Mast cells exposed to compound 48/80 (1 μg/ml) degranulated (Fig. $3A_2$) and released histamine (Fig. 3B). The cells became covered with granules. Most of these granules had disappeared after three washings in isotonic salt solution pH 8. Mast cells of fairly normal appearance then came into view. The semitransparent cell could be seen to contain granules, although fewer than originally (Fig. 3A). On a second exposure to compound 48/80 (1 μg/ml) the washed mast cells responded again with degranulation (Fig. $3A_4$) and histamine was released (Fig. 3B). Totally the second release of histamine was lower than the first one but when calculated as percentage of the prevailing histamine content of the cells at the moment of discharge the dose response curves to compound 48/80 were practically identical.

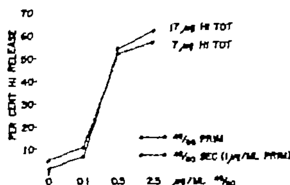


Fig. 3 B. Dose-response curves illustrating histamine release from isolated mast cells by two consecutive exposures to compound 48/80. The mast cells washed three times at pH 8 between the two exposures at pH 6.9.
Note Percentagewise the two dose-response curves are practically identical

It should be noted that if compound 48/80 is present in the wash fluid the mast cells do not recover their responsiveness to the releaser (Fig. 2)

B. Mast cells suspended in isotonic sucrose

Suspension of mast cells in hypertonic sucrose solution is deleterious to the ability of the cells to store and release histamine. During isolation by density gradient centrifugation in sucrose the mast cells therefore lose most of their histamine and the isolated cells do not respond to compound 48/80. Even suspension in isotonic sucrose solution unfavourably influences their storage and release mechanism. There occurs a progressive degranulation and histamine release and a successive decline in the sensitivity of the cells to compound 48/80. The storage and release abilities of the granules also decline when they are suspended in sucrose. The unfavourable effects of sucrose — probably due to dehydration of cells and granules will be described in a separate publication.

However, provided that the exposure of the mast cells to isotonic sucrose is brief and the pH of the sucrose medium optimal (around pH 7) the cells were found to respond to compound 48/80 with a degranulation, which — to judge from microscopical observations (Fig. 4A$_2$) — was of about the same magnitude as the degranulation occurring on exposure of the cells to compound 48/80 in an isotonic salt medium (Fig. 3A).

One difference was seen between the behaviour of granules discharged in isotonic salt solution and those discharged in sucrose pH 7. In sucrose solution the granules had much less tendency to stick to their mother mast cells or to aggregate into heavy clumps. They were dispersed in the suspension medium and could to a great extent be separated from the cells by differential centrifugation.

If the discharged cells were washed at pH 8 and resuspended in isotonic salt solution pH 7 (Fig. 4A) they responded again to compound 48/80 (Fig. 4A$_4$) as did the cells described under section A p. 5 indicating that the cells were not seriously deranged by the previous degranulation response in sucrose

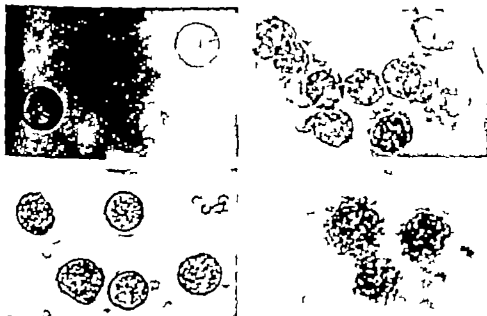


Fig. 4 A. Degranulation responses of isolated mast cells suspended in isotonic sucrose to two consecutive exposures to compound 48/80 (1 µg/ml) 37 C. Magnific × 990
1) Control cells in isotonic sucrose pH 7 (upper left)
2) Cells after 1st exposure to compound 48/80 (upper righ)
Note Less adhesion of gran les when cells re suspended in sucrose than when suspended in isotonic salt solution (Figs 3 A₂, 3 A and 4 A Picture 4 A₂ blurred because the cells were floating around in the sucrose solution)
3) Degranulated cells (4 A₂) washed three times in isotonic salt solution pH 7 8 10 min. 37 C (lower left)
Not The absence of adhering granules nd the rather normal appearance of the mast cells.
4) Degranulation of cells on 2 nd exposure to compound 48/80. Suspension fluid isotonic salt solution, pH 6.9 (lower righ)
Note The adhesion of extruded granules in contrast to A₂

Fig 5 compares the release of histamine from mast cells exposed to compound 48 80 in isotonic salt solution (left) and in isotonic sucrose solution (right) In isotonic salt solution the leakage of histamine in the control sample (O) was negligeable In isotonic sucrose sol tion the mast cells showed a slight spontaneous degranulation and roughly 10 per cent of the total histamine of the cell suspension was found in a granule fraction obtained on differential centrif gation (2, 00 × g) The mast cells showed a somewhat reduced sensit ity to compound 48 80 when suspended in sucrose the dose response curve being somewhat shifted to the right. The release could be normalized by adding calcium ions to the sucrose solution. However the addition of calcium ions caused release of histamine from the di harged granules.

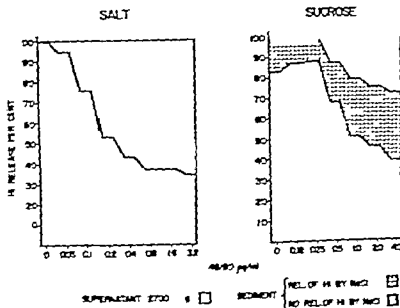


Fig. 5. Release by compound 48/80 of histamine from mast cells suspended in isotonic salt solution (left) and in isotonic sucrose solution (right). 3×10^4 cells/ml 37 °C 15 sec. pH 6.9.
☐ "Free" histamine (supernatant 2700 × g)
☐ Histamine in granules (sediment 2700 × g)
■ Histamine in intact mast cells (sediment 370 × g)

Comments

The noticeable difference between the response to compound 48/80 in salt and in sucrose solution was the occurrence in the sucrose solution of histamine-carrying granules. About 30 per cent of the discharged histamine was found in this granule fraction. The granule fraction was completely depleted of its histamine on suspension in isotonic sodium chloride solution.

The free histamine occurring in the supernatant both after water lysis and after degranulation caused by compound 48/80 in sucrose probably does not reflect any "free" histamine pool in the mast cell. This histamine fraction is probably formed outside the cell as a wash-out effect from the electrostatic binding in the granules as would be expected to occur if histamine was linked to acid groups such as carboxylic groups (Åborg et al. 1967).

Compound 48/80 induced release of histamine from mast cells and from isolated granules

Since compound 48/80 is a strong amine it has been argued that its releasing action on mast cells is due to a cationic exchange at the granule ionic sites between the releaser and histamine. However, the extrusion of histamine-containing granules from cells degranulated by compound 48/80 in isotonic sucrose solution does not favour this assumption. A further contra-argument is given in Fig. 6. Mast cells suspended in isotonic salt solution pH and histamine-containing granules from water lysed cells suspended in distilled water were both exposed to compound 48/80. Within the dose range used for our degranulation experiments (0.5—? μg/ml) compound 48/80 does not release any significant amounts of histamine from isolated granules. The effect of these doses of compound 48/80 is restricted to the triggering of the degranulation process, ionic exchange at histamine binding sites requiring supramaximal triggering doses.

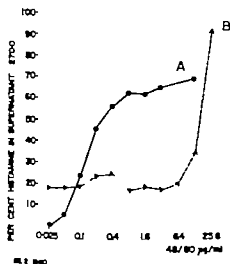


Fig. 6. Compound 48/80 induced histamine release from isolated mast cells ●—●—● and from isolated granules ▶---▶---▶. Mast cells suspended in isotonic salt solution pH 6.9, 37° C. Granules from water lysed mast cells suspended in distilled water.

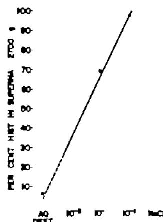


Fig. 7. NaCl-induced histamine release from granules isolated from mast cells degranulated in isotonic sucrose pH 7 by compound 48/80 (1 µg/ml), 37° C, 5 min.

Depletion, reuptake and redepletion of histamine from granules isolated from mast cells degranulated by compound 48/80

To study the storage mechanism of isolated granules mast cells suspended in isotonic sucrose were exposed to compound 48/80 (1 µg/ml) and discharged histamine-containing granules were collected by differential centrifugation (2,700 × g after an initial run at 370 × g to remove cells and coarse material). The granules were washed once in distilled water. Resuspension of these granules in a sodium-containing medium led to an immediate release of their histamine, the whole store being emptied with NaCl 10 M (Fig. 7).

In previous experiments (Thon and Uvnäs 1966) histamine-carrying granules obtained from water lysed cells were found to be depleted by suspension of the granules in the presence of heparin. Such granules were refilled when suspended in a histamine-containing but cation-free medium.

Granules isolated from mast cells exposed to compound 48/80 in isotonic sucrose were depleted of their histamine by suspension in heparin solution (500 µg/ml). The heparin was washed away with distilled water and the granules were resuspended in histamine-containing water. The pH was adjusted to 7 by mixing histamine base and histamine dihydrochloride. The granules were refilled (Fig. 8). The higher the extragranular histamine concentration the greater was the refilling.

Refilled granules released their histamine on suspension in NaCl-containing solutions just as they did with their original histamine.

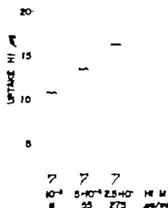


Fig. 8. Reuptake of histamine in histamine-depleted granules isolated from compound 48/80 degranulated mast cells.
Hatched bars: Histamine level in depleted granules before incub. with histamine.

Discussion

Our previous and present studies show that the mast cell histamine is stored in the granules in a rather weak electrostatic linkage allowing all the histamine to be released on exposure of the granules to cation containing media of sufficient strength i.e. those prevailing in body fluids. This fact may allow some speculations as to the relationship between degranulation and release.

If we postulate that histamine release from the granules is due to a cation exchange — and we have no reason to assume a more complicated mechanism — such an ion exchange can be imagined to occur *intracellularly* or *extracellularly*.

In vitro potassium has the same depleting action on the histamine store in the granules as has sodium. If the intracellular potassium ions had free access to the ionic binding sites in the granules the histamine storage mechanism should therefore break down more or less completely. However, there is one main difference between granules isolated *in vitro* and granules *in situ*. The former lack a surrounding membrane (Bloom and Haegermark 1965) but the latter have a membraneous cover belonging to the endoplasmic reticulum. This membrane has to be assumed to act as a shield against the penetration into the granules of intracellular cations.

Degranulation has been thought by many authors to be secondary to damage of the mast cell by the releasing agent (Paton 1956). No doubt many releasing agents increase the permeability of the mast cell membrane and subsequent intracellular osmotic disturbances will lead to swelling and disruption of the cells. Histamine will then undoubtedly be released from the granules by the action of intra- and extracellular cations.

However, the action of compound 48/80 on rat mast cells cannot have this simple explanation. After a degranulation response to this releaser the mast cell has still the

ability not only to store, without leakage, both granules and histamine but also to degranulate and release histamine on a second challenge with compound 48/80.

Together with the fact that the mast cell response required intact cell metabolism the observations best agree with the response being an active cell reaction.

It has been suggested that the histamine release should be secondary to an intra cellular cation exchange, the releasing agent or extracellular sodium being transported to the granular sites. Compound 48/80 is a strong amine and provided that it reached the granular sites in sufficient concentrations the amine should be able to compete successfully with histamine for the ionic sites in the granules. This would require a rapid accumulation of compound 48/80 in the granules after a passage against a concentration gradient through at least two membranes — the outer cell membrane and the gran le membrane. So far the penetration of compound 48/80 into the mast cell has not been demonstrated. There is another argument which seems to exclude an intracellular cation exchange behind the release of histamine. The intragranular concentration of histamine is ery high. If the histamine was uniformly distributed in the mast cell its concentration should amount to 0.4 M. If histamine were suddenly released from the granules by intracellular cation x change the intracellular concentration of histamine should rapidly reach values which would lead to cellular damage. It is highly improbable that the mast cells should remain functionally intact after being exposed to such high intracellular histamine concentrations.

Similar arguments to those used above can be used against the proposal that histamine release should be due to an intracellular cation exchange between extra cellular sodium and histamine. However the strongest argument gainst the idea of histamine release being dependent on an intracellular cation exchange is the fact that mast cells suspended in cation poor isotonic sucrose solution on exposure to compound 48/80 discharge *histamine-carrying* granules. Such gran les — which after their discharge lack a surrounding membrane (Bloom and Haegermark 1965) — are immediately depleted of their histamine the presence of cations g istonic sodium chloride. In their cationic exchange properties these discharged granules behave just granules isolated from non-discharged water-lysed mast cells, as previously reported by Thon and U nås 1966.

The histamine release in connection with degranulation response to compound 48/80 can thus best be explained as due to extracellula event, a cation exchange between histamine and sodium in the extracell lar fluid not requiring energy (Fig. 9)

The energy requiring part of the mast cell respons is the degranulation. It is easy to imagine that transport of granules to the surface of a cell requ res energy. It is more difficult to explain the intimate nat re of this transport mechanism. The granules are large particles, 0.1—0.5 μ in diameter. To us some kind of a mechanical transport due t a water pump system or contra tion process seem to be ttractive alternati hypotheses worthy of future investigations.

The mechanism by which compound 48/80 triggers the degranulation pro





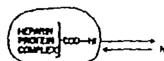


Fig. 9. Suggested mechanism of action of compound 48/80

still to be clarified. As a substance with a strong positive charge it might be able to cause electrostatic changes on the mast cell surface. The hypothesis that compound 48/80 triggers degranulation by removing an inhibitor or otherwise by activating some enzymatic step in a process leading to dregranulation is still to be proved or disproved.

Energy dependent mast cell responses are also initiated by other agents such as polypeptides, antigens to sensitized cells etc. For the same reasons as presented above the mechanisms of degranulation and release induced by these agents can be assumed to be in principle identical with those induced by compound 48/80.

In our views the mechanisms of degranulation and histamine release from mast cells might serve as a model applicable on other release processes. In the mast cell reaction the storage material — the heparin-protein complex — is actively discharged together with the stored amine, histamine. The amine is released by a simple ionic exchange process in the extracellular space. As was suggested previously (Uvnäs and Thon 1966) it might be a fruitful working hypothesis to assume a similar release mechanism of other amines e.g. at the nerve terminals, of polypeptides in endocrine gland cells, and of other electrically charged materials. Recent evidence in fact supports this hypothesis. Discharge of adrenaline from the adrenals has been shown to be accompanied by the output of both ATP and protein, the probable storage material in the catechol-carrying granules. Similar observations may be expected from studies of the release of other electrically charged substances stored in granules.

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Gastric and Duodenal Ulcers in Cats Following Reserpine

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Abstract

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The incidence of gastric and duodenal ulcers following reserpine was studied in cats. A single injection of 0.10 mg/kg of reserpine produced the highest incidence of antral ulcers (89 per cent) after 12 hrs and of duodenal ulcers (50 per cent) after 24 hrs. No ulcer was detected 8 days or more after the single injection. Reserpine injected every 24 hr for 2 to 4 days produced a lower incidence of antral ulcers than a single injection. In cats injected daily with reserpine for 6 days or more no ulcer was detected. Reserpine produced no ulcer in the oxyntic gland area. The ulcerogenic effect of reserpine remained after adrenalectomy. Truncal vagotomy or pretreatment with reserpine reduced the incidence of ulcers and also the acid response to reserpine. 0.10 mg/kg of reserpine was more effective to produce ulcer and to stimulate acid secretion than 0.03 mg/kg. The results suggested that the transient ulcerorgenic effect of reserpine was related to its effect on acid secretion.

The first observations that reserpine treatment might produce gastric and duodenal ulcers were made on man (Schroeder and Perry 1955, Humar and Bruno 1956). Reserpine induced ulcers in the stomach and duodenum have since been reported in different species (La Barre and Desmarez 1957, Blackman, Campion and Fastier 1959, Nicoloff *et al.* 1961) but the mechanism for the ulcerogenic effect of reserpine is unknown.

In studies on the mechanism by which reserpine reduces the antral and duodenal gastrin content in cats (Emås and Fyrö 1965) gastric and duodenal ulcers were detected in a great number of animals. The observation initiated further experiments on the ulcerogenic effect of reserpine. The present report describes the incidence of ulcers in cats following single and repeated injections of reserpine and the effect of truncal vagotomy and of adrenalectomy on the ulcerogenic effect. Most cats in this report have been used in the study referred to above.

Methods

Gastric and Duodenal Ulcer following Reserpine

Cats of both sexes (2.0 to 3.5 kg) were used. Animals subjected to two or more reserpine (Serpedin® Pharmacia) injections were fed with fish twice a day by hand if they did not eat

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by themselves. The cats were killed by air embolism or by rapid intravenous injection of sodium pentobarbital. No food or water was allowed for 24 hours before death. The stomach and duodenum were removed immediately after death and the mucosa was gently rinsed and examined macroscopically for ulcers. Pregnant animals and animals with food in the stomach were excluded. Microscopic examination was made of the antral mucosa from 4 cats injected with reserpine (0.10 mg/kg) 12 hrs before death and from 4 cats injected daily with reserpine (0.10 mg/kg) for 3 days and killed 24 hrs after the last injection. The specimens were fixed in formaldehyde (4 per cent) and the sections stained with hematoxylin and eosin, and with a combined elastin and van Gieson method.

A total of 111 cats received either a single i.m. injection of 0.10 mg/kg of reserpine and were killed 6 hrs to 16 days later or the same dose of reserpine was injected once a day for 2 to 8 days and the animals were killed 24 hrs after the last injection (Table I).

Ten cats received a single i.m. injection of 0.03 mg/kg of reserpine and 3 cats 2.0 mg/kg. They were killed 24 hrs after the injection.

Vagotomy was performed on 16 cats as described previously (Emås 1964). The truncal vagotomy was considered complete when insulin hypoglycemia produced no gastric acid response despite a blood sugar level of less than 50 mg glucose per 100 ml blood. Five cats were given a single i.m. injection of 0.10 mg/kg of reserpine and killed 24 hrs later; 3 cats were given the same dose of reserpine twice with 24 hrs interval and killed 24 hrs after the last injection. The remaining 8 cats received no reserpine and served as controls. All cats were killed within 1 to 2 months after the complete vagotomy.

Adrenalectomy was performed on 12 cats through a midline abdominal incision. Seven of the cats were given reserpine (0.10 mg/kg) 6 hrs after surgery and were killed 24 hrs after the reserpine injection. Five cats served as controls and were killed 30 hrs after the adrenalectomy.

Acid Secretion following Reserpine

Secretory studies were performed on 3 nonanesthetized cats provided with gastric fistulas (Emås 1960). They were deprived of food and liquid for 18 hrs before the experiment. The animals were given a daily injection of reserpine (0.10 mg/kg i.m.) for 2 consecutive days. Gastric secretion was recorded for one hour before and for 6 hrs after each injection of reserpine while the cats were standing in sling frames. Acid concentration was determined by titration against 0.01 N NaOH with phenolphthalein as indicator. The acid output is expressed in meq per hr. Two weeks or more elapsed between each two-day test and each cat underwent 3 such tests.

Results

Gastric and Duodenal Ulcer following Reserpine

Nonvagotomized Cats. The incidence of gastric and duodenal ulcers following 0.10 mg/kg of reserpine is shown in Table I. A single injection of reserpine produced in 6 hrs antral ulcers in 5 of 11 cats (45 per cent) and duodenal ulcers in 1 of 11 cats (9 per cent). The incidence of antral ulcers was highest, 8 of 9 cats, at 12 hrs after reserpine. The incidence then decreased and no antral ulcer was detected at 8 days, or later after a single reserpine injection (Fig. 1).

Following a daily injection of reserpine (0.10 mg/kg) for 2 to 4 days the incidence of antral ulcers was less than at 12 hrs to 4 days after a single injection of 0.10 mg/kg. No antral ulcer was detected in cats injected daily with reserpine for 6, 7 or 8 days (Fig. 1).

Reserpine in a dose of 0.03 mg/kg produced in 24 hrs one minor ulcer in 1 of 10 cats. All 3 cats that received 2.0 mg/kg of reserpine had multiple ulcers in the antrum 24 hrs later; a total of 18 ulcers were detected. In addition 2 of the 3 cats had ulcers in the proximal duodenum.

TABLE I Incidence of macroscopic ulcers in th trum nd duodenum of nonvagotomized cats following a singl or a daily injection of reserpine (0.10 mg/kg i.m.)

Time for sacrifice in days after 1st reserpine injection	Number of reserpine injections	Number of cats	Number of cats with ulcers in Antrum	Number of cats with ulcers in Duodenum	Total number of ulcers in Antrum	Total number of ulcers in Duodenum	Distribution of ulcers in cm from the pyloric sphincter: Antrum	Distribution of ulcers in cm from the pyloric sphincter: Duodenum
6/24	1	11	5(45 %)	1(9 %)	23	1	2.5	1 0
9/24	1	5	3(60 %)	2(40 %)	23	3	3.0	0 5
12/24	1	9	8(89 %)	3(33 %)	35	6	3.0	4 0
1	1	22	13(59 %)	11(50 %)	28	17	3.0	4 0
2	1	3	2(67 %)	0	3	0	1.5	—
4	1	5	3(60 %)	2(40 %)	6	2	2.0	1.0
6	1	6	2(33 %)	1(17 %)	2	1	1.5	1.0
8	1	3	0	0	0	0	—	—
12	1	3	0	0	0	0	—	—
16	1	3	0	0	0	0	—	—
2	2	8	4(50 %)	2(25 %)	12	4	2.5	2.0
3	3	10	5(50 %)	1(10 %)	9	1	3.0	0.5
4	4	9	3(33 %)	0	3	0	1.0	—
6	6	3	0	0	0	0	—	—
7	7	2	0	1(50 %)	0	1	—	1.0
8	8	9	0	0	0	0	—	—
0 (controls)	0	50	0	0	0	0	—	—

Cats that received repeated injections were sacrificed 24 hrs after last injection.

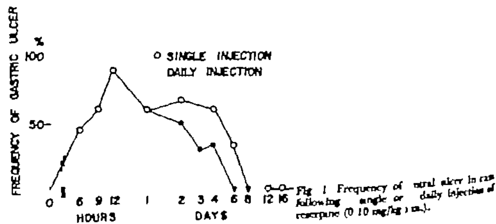


Fig 1 Frequency of ntral ulcer in cats following ngle or daily injection of reserpine (0 10 mg/kg i.m.).

Vagotomized Cats. None of 5 cats tested had gastric or duodenal ulcer at 24 hrs after a single injection of reserpine (0.10 mg/kg). Reserpine daily for 2 days produced a small (1 mm in diameter) antral ulcer in 1 of 5 cats.

Adrenalectomized Cats. A single injection of reserpine (0.10 mg/kg) produced in 4 hrs multiple antral ulcers (2—5 mm in diameter) but no duodenal ulcers, in the 7 cats tested.

Five cats died during the reserpine treatment. They were excluded from the study since the time of death could not be established.

No gastric or duodenal ulcer was detected in 50 nonvagotomized cats, 5 of which were adrenalectomized, and in 8 vagotomized cats that either had received no injection or had been injected with the vehicle for reserpine.

Most ulcers following reserpine were narrow and linear (up to 5 mm long) and a few circular (diameter up to 3 mm). All gastric ulcers appeared within 3 cm from the pylorus, i.e. in the antral part of the stomach. A few large ones had perforated the entire stomach wall. Ulcers in the duodenum were less frequent than in antrum. They were localized in the proximal 4 cm of the duodenum. The number of ulcers is given in Table I.

Microscopic examination of the antral mucosa at 12 hrs after reserpine showed moderate acute inflammatory reaction and oedema around the ulcers. Most ulcers were superficial although ulcers were found that extended to or partly penetrated the muscularis mucosae and were associated with bleeding. In a few superficial ulcers and erosions the epithelial cells varied in size and shape, and had rather large nuclei rich in chromatin. This was interpreted as regeneration had started. Signs of regeneration were lacking or sparse around the deep ulcers. In cats treated with reserpine for 3 days extensive acute inflammatory reaction was seen around the deep antral ulcers. Most superficial and all deep ulcers were surrounded by regenerating mucosa.

Acid Secretion following Reserpine

In 9 expts. on 3 cats with gastric fistulas the average basal output of acid was less than 0.02 meq per hr (Table II). Reserpine (0.10 mg/kg i.m.) produced a gradual increase in acid output after 30 to 60 min. Peak output occurred in the 3rd post-injection hour. Acid secretion then remained rather constant throughout the subsequent 3 hrs. The mean 1-hr output of acid during the 3rd through 6th h. for each cat is given in Table II.

Basal secretion of acid was elevated 24 hrs after the first injection of reserpine and ranged in the cats from 0.16 to 0.54 meq per hr (Table II). Reserpine stimulated acid secretion also when the cats were pretreated with reserpine but the response was smaller than that to the first injection given the day before. The mean 1-hr output of acid during the 3rd through 6th h. to the second injection of reserpine was 35, 43 and 49 per cent lower in the individual cats than the response to the first injection given 24 hrs earlier (Table II). According to ordinary methods for analysis of variance (Snedecor 1956) the difference in acid response to the first and second injection was significant ($p < 0.01$).

TABLE II Mean acid output in nonanesthetized gastric fistula cats before and after 1st and 2nd injection of reserpine (0.10 mg/kg i.m.) given with 24 hrs interval

Cat	Number of expts.	Mean acid output in meq/hr				Difference in acid output to 1st and 2nd injection	
		1st day of exp.		2nd day of exp.			
		Basal	After reserpine	Basal	After reserpine[1]	1 meq	In per cent
A	3	0 02	*3.18* 2.60—3.56[2]	0.54	*2.08* 1 41—3.10	*1 10*	35
B	3	0 02	*2.93* 1 79—3.83	0.35	*1.63* 1 13—2.24	*1.30*	44
C	3	0 02	*3.61* 2 62—4.32	0.16	*1.84* 1 16—2.71	*1.77*	49

[1] Mean 1 hr acid output during the 3rd through 6th hr after reserpine
[2] Range

The mean concentration of acid in the responses of individual cats during the 3rd through 6th hour after the first and second injection of reserpine was 162 and 158 meq per liter respectively in cat A, 146 and 150 meq per liter in cat B, and 159 and 140 meq per liter in cat C. The difference in acid concentration following the first and second reserpine injection was not significant ($p>0.05$)

Discussion

In the present study a single injection of reserpine (0 10 mg kg) produced in 12 hrs gastric ulcers in about 90 per cent of the cats with intact vagus nerves. All gastric ulcers were located in the antrum. One day after reserpine the incidence of antral ulcers was about 60 per cent. The ulcer incidence further decreased during subsequent days and after 6 to 8 days no ulcer was found whether the cats had received either a single or a daily injection of reserpine. Reserpine produced ulcers also in the proximal duodenum, but less frequently than in the antrum. The highest incidence of duodenal ulcer was obtained 24 hrs after reserpine when 50 per cent of the cats had ulcers. No duodenal ulcer was detected 8 days after a single or a daily injection of reserpine. Ulcers were never seen in the oxyntic gland area.

The incidence of antral ulcers decreased from 90 per cent at 12 hrs after a single injection of reserpine to 30 per cent at 3 days after the first injection, despite the fact that reserpine was injected daily. The first injection, therefore appeared to be the most important for the production of ulcers. The decrease in ulcer incidence also suggested a rapid healing of most ulcers, and a great regenerative capacity of the gastric mucosa (Grant 1945)

Vagotomy almost blocked the ulcerogenic action of reserpine a small ulcer was detected in 1 of 8 vagotomized cats injected once or twice with 0.10 mg kg of reser-

pine. Similar observations have been made in mouse (Blackman *et al.* 1959 d rat (Kim and Shore 1963) and suggest that the ulcerogenic effect of reserp ne is dependent on intact vagus nerves.

Studies on the dog (Smith and Howes 1964) mouse (Blackman *et al.* 1959) and rat (Chan Jou Chu 1959 Damrau 1961) indicate that acid secretion is essential for the development of ulcers following reserpine. In the present study the amount of acid secreted and the incidence of ulcers following reserpine appeared to run parallel. Reserpine in a dose of 0.05 mg/kg, produced a smaller acid response (*cf* Emås 1965) and a considerably lower incidence of ulcer than a dose of 0.10 mg/kg. The first injection of reserpine (0.10 mg/kg) produced a larger acid response (Table II) and a higher incidence of ulcer (Table I) than a second injection given 24 hrs later th acidity of the responses did not differ. Finally truncal vagotomy reduced the acid response to reserpine (Emås 1965) and the incidence of ulcer.

Although the amount of acid secreted following reserpine ppears to be important for the development of ulcer the precise mechanism for the ulcerogenic action of reserpine is not known. The complex action of reserpine in olves several components which might be implicated in the development of ulcer. For example reserpine reduces the gastrin content in the vagally innervated but does not alter the content in the vagally denervated, antral and duodenal mucosa (Emås and Fyrö 1965). The reduction is presumably due to a vagal release of gastrin. A continuous i.v. infusion of exogenous gastrin for 24 hrs has rece tly been shown to produce ulcers in cats (Emås and Grossman 1967) but the ulcers nduced by gastrin, in contrast to those induced by reserpine, were mainly located in the duodenum. Released gastrin as the only mediator for the ulcerogenic action of reserpine, therefore, appears unlikely. In addition to the reduction of the gastrin content reserpine causes an increased sensitivity of the HCl secreting cells to gastri (Emås 1963). Also the latter effect of reserpine is dependent on intact gus nerves (Emå 1964). The low incidence of reserpine induced ulcers after agotomy was probably related to the low acid output in the agotomized cats which in part could be attributed to the elimination both of vagal gastrin release and of the gal sensitizing effect on the HCl secreting cells.

A single injection of reserpine reduces the ACTH content of the pituitary and elevates the plasma corticosteroid level (Egdahl Richards and Hume 1956, Kitay Holub and Jailer 1959 Maickel Westermann and Brodie 1961). Our finding that reserpine produced antral ulcers in adrenalectomized cats indicated that the ulcerogenic action of reserpine was not dependent on pit itary-adrenal mechanisms.

Reserpine causes a depletion of histamine and serotonin from different organs (Pletscher Shore and Brodie 1955 Ha erback and Wertschafter 1962, Kim nd Shore 1963) and of catcholamines from adrenergic nerve terminals (ref in Emås 1963). Both histamine (Hay *et al.* 1942) and serotonin (Haverback and Bogdanski 1957) ha e ulcerogenic action and the lcerogenic action of histamine is reported to be enhanced by drenalin (Leonard *et al.* 1961). To what extent these bio amines are implicated in the reserpine lcerogenic action of reserpine remains elucidated.

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Influence of Vasoconstrictor Nerve Activity on the Cholinergic Vasodilator Response in Skeletal Muscle in the Dog

By

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Abstract

Bolme, P S. H. Ngai and S. Rosell. *I fluenc of vasoconstrict ce activity on the cholinergic vasodilato esponse in skel tal muscle in the d g* Acta physiol. scand. 1967 *71* 323—333

The cholinergic sympathetic asodilator nerves were acti ted by topical stimulation i the hypothalamus and the mesencephalon during graded levels of socontrictor nerv activity Chloralose anesthetized as well as conscious dogs were used I the anesthetized dogs the isolated gracilis muscle was perfused with blood t constant flow rate. The perfusion pressure as recorded. I unanesthetized dogs blood flow was measured with an lectromagnetic flowmeter probe placed around the external iliac artery Increase in asoconstrictor nerv ac ivity as produced either by clamping one or both common carotid arteries or by central topical stimulation through electrodes in the vasoconstrictor rea in the hypothalamus. Acti tion of cholinergic asodilator nerves produced vasodilator response in the skeletal muscles. An increase in asoconstrictor tone inhibited the effect of threshold asodilator nerv stimulation. However when the asodilator nerves were stimulated with suprathreshold ensity the asodilator response could be produced even at an elevated asoconstrictor tone On the other hand very pronounced asoconstrictor tone only possible to produce in the anesthetized dogs, reduced the vasodilator response in some experiments The experimental find gs indicate peripheral antagonism between the two types of vasomotor nerves, however t least i dogs this is presumably of minor physiological importance A strong activ tion of holinergic asodilator nerves may increase the muscle blood flow even f he rterioles re nder ncreased asoconstrictor nerv activity

The skeletal muscle blood vessels are nnervated by drenergi soconstr ctor and cholinergic vasodilator fibres. The asoconstrictor fibres nerv t d fferent consecutiv sections of the ascular bed, prec pillary resistance essel (rterioles) precapillary sphincters nd post pillary resistance nd capacitanc essels, wherea the asodilator nerves seem t inners t onl the prec pillary resist nc essel (Mellander 1960, Folkow Mellander nd Obe g 1961 Rosell d I na 1962 Cobbold *et l.* 1963 These essel are thus innervated by two type f asomotor nerves.

There are ery few investigations lucidat ng the teraction between these two types of sympathetic asomotor nerves There the possil l ty of either antagonism or synergism between the two systems When they re ctiv ted contemporaneously which is the case during sympathetic hai st n lation v onstric

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tion is usually induced. However the vasoconstriction is sometimes preceded by an initial vasodilation due to vasodilator nerve activity (Folkow and Uvnäs 1948). In other studies the sympathetic vasodilator outflow was stimulated electrically in the central nervous system during bilateral common carotid occlusion after vagotomy. It was reported that the higher the prevailing vascular tone the greater was the increase in blood flow produced by stimulation (Eliasson, Lindgren and Uvnäs 1952, Lindgren and Uvnäs 1953). Lindgren (1955) compared on cat the response produced by stimulation of the sympathetic vasodilator outflow in the tectum mesencephali before and during bilateral common carotid occlusion in vagotomized cats. The vasodilator response did not seem to be influenced by the increased vasoconstrictor tone produced by common carotid occlusion (see Fig. 52 in Lindgren 1955). On the other hand, Folkow, Öberg and Rubinstein (1964) stimulated electrically the vasodilator outflow in the hypothalamus in cats and compared the effects during high and low vasoconstrictor nerve activity. They found that the vasodilator response was very much influenced by the prevailing vasoconstrictor tone in skeletal muscle vessels. At a high steadily maintained tone the vasodilator response was more or less completely supressed. These experiments thus indicate that the vasodilatation induced by vasodilator nerve activity may be of physiological significance only during low vasoconstrictor tone.

Since Folkow and coworkers were primarily interested in elucidating the peripheral neuro-effector organization of the two vasomotor systems, they produced clearcut, sustained vasoconstrictor effects to study their influence on the vasodilator nerve responses. The question still remains, however, whether or not the blockade of the vasodilator response is of importance even at ranges of vasoconstrictor nerve activity which are supposed to occur during physiological conditions. For this reason the present study on conscious and anesthetized dogs was performed.

Methods

The experiments were performed on mongrel dogs, weighing 10—18 kg, some of which were anesthetized and some studied in the conscious state. The blood flow was measured either to the muscles of the hind limb or to the intact hind limb. In one series of experiments the isolated gracilis muscle was perfused with blood at constant flow rate.

For experiments in the conscious state four dogs were prepared by operative procedures. Under pentobarbital anesthesia (25 mg/kg [illegible]) laparotomy was performed and a polyethylene catheter was inserted into the abdominal aorta. A previously calibrated electromagnetic flowmeter probe of appropriate internal diameter was fitted around the external iliac artery. The catheter and the leads from the probe were threaded through the posterior abdominal wall to the back to emerge in the interscapular region. To allow temporary arterial occlusion to estimate the zero point of blood flow tracings, a silk loop was placed around the iliac artery distal to the probe emerging in the groin. Carotid loops were prepared by placing the common carotid arteries in subcutaneous tunnels.

After an interval of 2—4 days each animal was again anesthetized with chloralose (100 mg/kg i.v.) and monopolar electrodes were inserted stereotaxically into the vasodilator area in the hypothalamus or the mesencephalon. When appropriate responses following electrical stimulation (i.e. an increase in blood flow to the hind limb) was obtained the electrodes were fixed with acrylic cement and connected to external leads. The electrical earth was provided by a screw fixed to the skull. In two of the dogs electrodes were also implanted in structures in the hypothalamus where stimulation evoked predominantly pressor responses.

Experiments were carried out after recovery from the second operation. The circulatory responses to activation of the vasodilator outflow were compared at high and at low

constrictor nerve activity Increase of vasoconstrictor nerve activity was produced either by clamping the common carotid arteries or by stimulating the vasoconstrictor outflow in the hypothalamus through the implanted electrode.

Stimulus current consisting of square pulses with a frequency of 80 cps, pulse duration of 2 msec and varying intensities were obtained from Grass Model S 4 D stimulator The arterial pressure was measured in the aortic catheter connected to Statham transducer (P 23 AC) A air wave electromagnetic flowmeter (AB Eledia Stockholm) measured the external iliac arterial blood flow The heart rate was recorded with an ordinate writer fed with either the pressure or the flow signal (Goldschmidt and Lindgren 1962) All recordings were made on Grass Model 5 Polygraph. Vascular resistance was calculated from the mean arterial pressure and the blood flow Atropine (0.1—0.3 mg/kg i.a.) was given to every dog to test the nature of the vasodilatation obtained. The methods used in the conscious dogs are described in more detail by Bolme *et al.* (1967)

The same type of experiments as described above were also performed on five chloralose anesthetized dogs (100 mg/kg i.) Blood flow either to the whole hind limb or after skinning, to the muscles of the hind limb was recorded as above Increased vascular resistance was obtained by common carotid occlusion before and after vagotomy Cholinergic vasodilator response was produced by hypothalamic or mesencephalic stimulation.

Fourteen experiments were carried out on morphine pretreated (10 mg/kg s.c.) and chloralose (100 mg/kg i.) anesthetized dogs. The gracilis muscle was isolated and perfused from reservoir filled with the dog own blood after heparinization. The flow was kept constant (Renkin and Rosell 1962) Increased sympathetic vasoconstrictor tone was produced by common *carotid occlusion. The vagus nerves were either left intact or cut in the cervical* region. Sympathetic vasodilator response was obtained by hypothalamic stimulation. The systemic arterial pressure and the perfusion pressure t the gracilis muscle were measured by Statham transducers (P 23 AC) I some experiments the effects of vasoconstrictor nerve activity were blocked by injection of dihydroergotamine (0.1 mg i.a.) The efflux from the muscle as cannulated and the outflow measured by means of silicone filled drop counter Recordings of the perfusion pressure and the systemic pressure were made on Rikadenki multipen recorder or on Grass Model 5 Polygraph.

Results

In five chloralose anesthetized dogs blood flow to the intact hind limb, or to the hind limb muscles was recorded and the sympathetic vasodilator outflow was stimulated electrically in the brain stem. The effects of the stimulation during prevailing low vasoconstrictor tone and during common carotid occlusion were compared. Following bilateral vagotomy the increase in vasoconstrictor tone during common carotid occlusion was more pronounced. Fig 1 Illustrates one such experiment. The response to supramaximal stimulation of the hypothalamic vasodilator outflow when the peripheral resistance was relatively low (1 1) is shown in the left panel. *The blood flow increased from 85 ml/min to 140 ml/min and the* arterial pressure nd heart rate increased somewhat. The peripheral resistance value decreased to 0.9 In the middle panel, common carotid occlusion increased the vasoconstrictor tone the peripheral resistance value rose from 1 1 to 2.2 When the hypothalamic stimulation was repeated the blood flow increased to 150 ml/min. The arterial pressure and the heart rate increased as well. The peripheral resistance value decreased t 1 2. Thus, the increase in total blood flow was more pronounced on stimulation from a high resting peripheral resistance level than from a lower one However as indicated by the resistance value which pon stimulation of the vasodilator outflow was 0.9 during the control and 1.2 during common carotid occlusion, the increased vasoconstrictor tone was not totally overcome by activation of the sympathetic vasodilator system. Further in one of these five experiments the response to suprathreshold stimulation of the hypothalamic vasodilator outflow

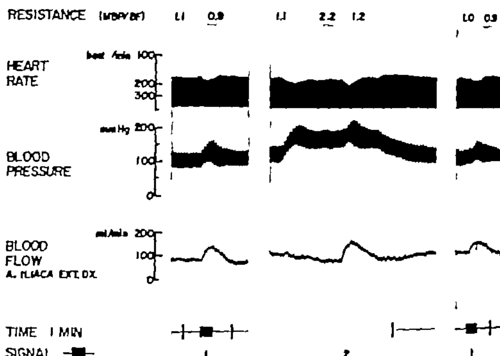


Fig. 1. Dog 15 kg. Chloralose. Bilateral vagotomy. Skinned leg. Effect of stimulation of vasodilator pathways in the hypothalamus.
1. Stimulation with 3.5 V during resting vasoconstrictor nerve activity.
2. Stimulation with 3.5 V during bilateral common carotid occlusion indicated by interruption of the event marker.
Vascular resistance (R) was calculated as mean blood pressure (MBP)/blood flow (BF).

was markedly reduced when the vasoconstrictor tone, produced by common carotid occlusion, was elevated to an almost maximal level. This is illustrated in Fig. 2 where the effect of vasodilator stimulation was influenced only to a minor extent when the vasoconstrictor tone was moderately elevated by common carotid occlusion with the vagus nerves intact (A). On the other hand, when the same stimulation was repeated after bilateral vagotomy (B) the resulting extensive vasoconstrictor tone clearly inhibited the vasodilator response. In this experiment the increase in peripheral resistance in the skeletal muscles upon common carotid occlusion is probably underestimated since it was performed on an intact hind limb. It has been shown that the increase in peripheral resistance due to a decrease in baroreceptor activity is preferably confined to the skeletal muscle vessels whereas the vessels of the skin and subcutaneous adipose tissue are involved to a much lesser extent (Löfving 1961, Ngai, Rosell and Wallenberg 1966).

These experiments thus indicate that the vasodilation produced by vasodilator nerve stimulation can overcome a moderate increase in vasoconstrictor tone. However a maximal increase in constrictor tone seems to counteract the vasodilator response.

Experiments in the conscious state were performed in four dogs. Stimulation of

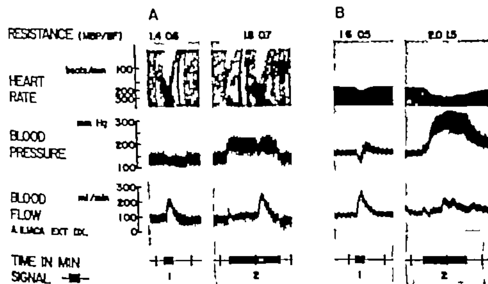


Fig 2. Dog 13 kg. Morphine-Chloralose Effect of stimulation of vasodilator pathway in the hypothalamus.
1 Stimulation with 1.5 V during resting vasoconstrictor nerve activity
2 Stimulation with 1.5 V during bilateral common carotid occlusion.
Between A and B Bilateral vagotomy The dilatation following stimulation was completely blocked by atropine (0.2 mg/kg i.a.) not shown in the picture.

the vasodilator outflow was made with currents of suprathreshold intensity Increased vasoconstrictor tone was either produced by common carotid occlusion or by stimulation via an electrode implanted in the pressor area of the hypothalamus. Since the vagus nerves were intact, drastic increases in vasoconstrictor tone could not be obtained, probably due to homeostatic regulatory mechanisms counteracting the pressor response. In Fig 3 one typical experiment is illustrated. Stimulation of the hypothalamic vasodilator outflow during the control condition increased the blood flow from 70 ml/min to 135 ml/min. Heart rate and arterial pressure increased as well. The peripheral resistance dropped from 1.3 to 0.8. During common carotid occlusion the peripheral resistance value increased to 2.4 When the stimulation was repeated the blood flow to the hind limb increased from 60 ml/min to 130 ml/min and the resistance value decreased to 1.1 The results thus agree with those on the chloralose anesthetized dogs. Suprathreshold stimulation of the sympathetic vasodilator outflow produced an increase in blood flow of comparable magnitude during resting conditions and during states of moderately elevated vasoconstrictor tone. On the other hand, this could apparently not be totally overcome which is indicated by the fact that the resistance reached lower values following vasodilator nerve stimulation during resting conditions than during elevated vasoconstrictor tone

In order to study the interaction between the two vasomotor systems in a more quantitative manner experiments were performed on 14 dogs with the gracilis

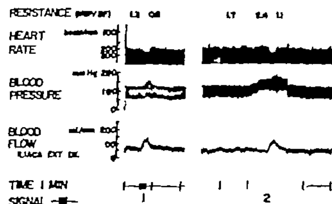


Fig. 3. Dog 15 kg. Conscious. Effect of stimulation of vasodilator pathways in the hypothalamus. 1 Stimulation with 4 V during resting vasoconstrictor nerve activity. 2. Stimulation with 4 V during bilateral common carotid occlusion.

muscle isolated except for its nervous supply and perfused with blood at a constant rate. The sympathetic vasodilator nerves to the muscle were activated by hypothalamic stimulation. Fig. 4 shows one of these experiments. Electrical stimulation of the hypothalamic vasodilator outflow (A) with currents of submaximal intensity 1.75 V was followed by a decrease in the perfusion pressure from 150 to 90 mm Hg. Following stimulation with a higher intensity, 2 V, which in this experiment gave a maximal response, the perfusion pressure decreased from 135 to 70 mm Hg. The systemic arterial pressure increased following stimulation and there was a certain latency until the perfusion pressure returned to the prestimulatory level. Common carotid occlusion (B) increased the perfusion pressure as well as the arterial pressure. Repeated stimulation with 1.75 V resulted in a drop in perfusion pressure of 15 mm Hg during the stimulation (indicated on the curve by vertical lines). The

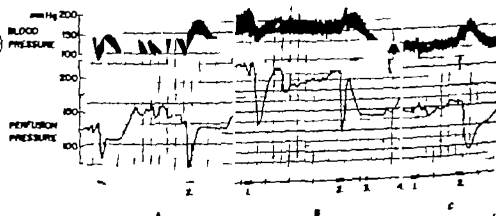


Fig. 4. Dog 11 kg. Morphine-Chloralose. Bilateral vagotomy. Constant blood flow perfusion of M. gracilis (10 ml/min/100 g). Effect of stimulation of vasodilator pathways in the hypothalamus.
A. Resting conditions. B. Bilateral common carotid occlusion. C. After atropine.
1. Stimulation with 1.75 V.
2. Stimulation with 2 V.
3. End of common carotid occlusion.
4. Atropine i.a. 0.1 mg.
The duration of the stimulation is indicated by the small vertical lines on the perfusion pressure curve.



Fig 5. Dog 14 kg Morphine-Chloralose. Bilateral vagotomy Constant blood flow perfusion of M. gracilis (5 4 ml/min/100 g) Effect of stimulation of vasodilator pathways in the hypothalamus.
1 Stimulation with 1 75 V
2 Stimulation with 2 V
3 Atropine i.a. 0 1 mg.
The duration of the stimulation is indicated by the small vertical lines on the perfusion pressure curve.
Note the two stage dilatation on stimulation before atropine. The first stage was blocked by atropine, the second remained unchanged.

vasodilator effect was thus markedly reduced as compared with the effect of stimulation when the vasoconstrictor tone was low A small increase in the systemic arterial pressure and a secondary pronounced poststimulatory decrease in perfusion pressure were also seen following the stimulation. However when stimulation was made with the higher intensity 2 V the perfusion pressure decreased during the stimulation period from 205 to 120 mm Hg The vasodilator activity could thus overcome the vasoconstrictor tone. On the other hand, as in the experiments related above, the increased vasoconstrictor nerve activity was not totally overcome since had that been the case the perfusion pressure should have decreased to about 70 mm Hg (as shown in A) This is in contrast to the finding in many of the experiments that intra-arterial injection of acetylcholine always produced a maximal vasodilatation whether a low or a high vasoconstrictor tone was prevailing (not shown in the fig) After atropine (C) no vasodilatation was produced during the stimulation. However with 2 V a vasodilatation was still observed after cessation of the stimulation.

The poststimulatory vasodilatation was an almost regular finding in experiments where a relatively high peripheral resistance prevailed. Fig 5 shows an experiment on the isolated gracilis muscle where the resting vascular resistance was about twice as high as in the experiment shown in Fig 4 indicated by the lower blood flow at about the same perfusion pressure Stimulation of the hypothalamic vasodilator outflow with 1 75 V for 10 sec produced a decrease in the perfusion pressure of 30 mm Hg. A slight increase in the systemic arterial pressure occurred following the stimu-

lation. Further about a minute elapsed before the perfusion pressure returned to the prestimulatory value. With 2 V a decrease in perfusion pressure of 40 mm Hg was produced during the stimulation. A pronounced rise in the arterial pressure was elicited and upon cessation of the stimulation a marked second drop in perfusion pressure appeared. When the stimulation was repeated after administration of atropine the vasodilator response during the stimulation was blocked. However stimulation with both 1.75 V and 2 V increased the arterial pressure and the post-stimulatory vasodilatation was not influenced by atropine. The poststimulatory vasodilatation could be blocked by dihydroergotamine and furthermore, the higher the increase in arterial pressure, the more prominent was the poststimulatory vasodilation. These findings indicate that the secondary vasodilatation was caused by inhibition of the vasoconstrictor tone, probably resulting from baroreceptor excitation due to the increased arterial pressure.

Discussion

The present study shows that it is possible to induce a vasodilator response in skeletal muscle vessels by stimulation of the cholinergic vasodilator nerves during a low as well as an increased vasoconstrictor activity. However there seems to be a certain degree of antagonism between the vasodilator response and the vasoconstrictor tone. This was most evident when vasodilator stimulation was performed with a low stimulus intensity. Under these circumstances the response could almost be abolished by an increased vasoconstrictor nerve activity. Furthermore, vasodilator responses even following suprathreshold stimulus intensities could be markedly reduced by extreme elevation of the vasoconstrictor tone. The fact that the increase in blood flow in ml/min was often greater when stimulation of the vasodilator nervous outflow was performed from a level of high than from a low vasoconstrictor tone does not contradict this statement since an increase in the blood flow (or a decrease in the perfusion pressure at constant flow rate) may not give a true indication of the degree of relaxation of the arteriolar smooth muscles. This is illustrated by the experiment in Fig. 4. When stimulation was performed during the lower level of constrictor tone the perfusion pressure decreased 65 mm Hg (from 135 to 70 mm Hg), whereas following stimulation during the higher level of vasoconstrictor tone the perfusion pressure decreased 85 mm Hg (from 205 to 120 mm Hg). This may give the impression that the dilatation in the second case was greater than in the first one.

To give a more direct estimation of the dilatation the change in mean radius of the vessels may be calculated according to Poiseuille's law[a] by which the mean radius of the resistance vessels can be estimated as the fourth root of the inverted resistance, also provided the fluid viscosity and the length of the vessels are constant. The change in mean radius can be determined by assuming that the radius was 1 at maximal dilatation, i.e. when the perfusion pressure reached the value of 70 mm Hg. At stimulation with 2 V during rest the mean radius then increased from $\sqrt[4]{\frac{7}{13.5}} = 0.85$ to 1, an increase of 17 per cent. At stimulation during high constrictor nerve activity the radius increased accordingly from $\sqrt[4]{\frac{7}{20.5}} = 0.76$ to $\sqrt[4]{\frac{7}{12}} =$

= 0.87 an increase of 14 per cent. This example thus illustrates that even if the vasodilator response expressed in mm Hg (or ml/min) seems to be greater from state of high resting tone than from lower one the opposite may be the case when the vasodilatation is calculated as change in relaxation of the arteriolar smooth muscles.

The experimental results on the isolated gracilis muscle were all calculated with respect to change in radius. In four experiments the increase in radius was greater when the vasoconstrictor tone was elevated, in 8 expts. the opposite was evident, and in the remaining two experiments no difference was observed. Thus, these data indicate that the increase in the vasoconstrict r nerve activity does not necessarily exert an inhibitory effect on the relaxation of the smooth muscles following vasodilator nerve stimulation. On the other hand, a ery pronounced increase in vasoconstrictor tone inhibited the vasodilator response (see Fig ?) However it was often difficult to elevate the vasoconstrictor tone to such an extent that the vasodilator response was much reduced especially in the conscious animals with all barostatic mechanisms intact. This may be the reason why in the conscious animals the increased vasoconstrictor tone never reduced the vasodilator response to any significant extent. In experiments where the aortic baroreceptor mechanisms were excluded by vagotomy it was possible in only three out of 19 expts. to demonstrate a clearcut inhibition of the vasodilator response by a very high vasoconstrictor tone

These results apparently are at variance with those reported by Folkow Öberg and Rubinstein (1964) in cats. These authors observed a much more pronounced supression of the vasodilator response when the vasoconstrictor activity was enhanced. In representative experiment, sympathetic vasodilator timulation during control conditions decreased the peripheral resistance alue 3—4 times. When the vasoconstrictor tone was elevated, the same vasodilator stimulation resulted in an almost insignificant vasodilator response The difference in results may be quantitative rather than qualitative since the proportion of vasodilator to vasoconstrictor fibres might be greater in the dog than in the cat. A finding in fa our of such a

$Q = \frac{\Delta P \; r^4 \; \pi}{\mu \; 8 \; l}$ Q = flow P = pressure, = radius of the "tube μ = viscosity l = length of the "tube

quantitative difference between the cat nd the dog may be the two-stage dilatation illustrated in Fig 5 The experiment showed that activation of the sympathetic cholinergic vasodilator outflow produced an immediat response in spite of a rather high prevailing vasoconstrictor tone The stimulation also resulted in an increased systemic arterial pressure which pparently led to inhibition of the vasoconstrictor tone resulting in the second phase of the dilatation which was not blocked by atropine It is of importance to not that the immediately-occurring vasodilatation seems t be entirely due to activation of vasodilator nerves and not to a combined effect of vasodilator nerv activ ty and inhibition of vasoconstrictor tonus. The evidence for this is that after atropine the immediately-occurring vasodilatation was completely blocked. In the cat Folkow Öberg and R binstein (1964) also found a two-stage dilatation following sympathetic vasodilator stimulation at a level of high resting soconstrictor tone The first stage of the dilatation was m

phase with a rise in arterial pressure. However after a delay of 8—12 sec from the onset of the stimulation a sudden increase in blood flow appeared which was atropine sensitive. The authors suggested that the centrally induced rise in the arterial pressure led to activation of the baroreceptors resulting in a reflex inhibition of the vasoconstrictor tone which had to develop before the vasodilator fibres could dilate the resistance vessels. This apparent difference between the cat and the dog may be expected if the skeletal muscles in the cat have a relatively richer innervation of vasoconstrictor fibres as compared with the dog. If this is the case, a high vasoconstrictor tone must first be reduced before a vasodilator nerve activity in the cat exert its effects while in the dog this is not necessary.

The suggestion of a quantitative species difference might also be supported by the fact that as far as the vasodilator nerves are concerned there are certain species differences. Bolme, Novotny, Uvnäs and Wright (to be published) have found that several species such as monkeys, polecats, badgers and hares seem to lack vasodilator nerves whereas the existence of such nerves have been demonstrated in foxes, sheep and goats besides cats and dogs.

However the difference in results between Lindgren (1955) and Folkow, Öberg and Rubinstein (1964), both studies performed on cats, still remains to be explained. Lindgren (1955) observed that the response to mesencephalic vasodilator activation seems to be independent of the prevailing level of vasoconstrictor tone. Lindgren presumably activated the vasodilator fibres rather specificly as indicated by the absence of a pressor response. Folkow, Öberg and Rubinstein (1964) produced by stimulation, in addition to the increase in muscle blood flow due to vasodilator nerve stimulation, a pronounced rise of the arterial pressure indicating a concomitant activation of vasoconstrictor fibres not only to cutaneous and intestinal areas but probably also to the skeletal muscles studied. Thus, the experiments by Folkow, Öberg and Rubinstein (1964) may be compared with those of ours in which the increase in vasoconstrictor tone was especially high and in which we also saw a reduction of the vasodilator response. The experiments by Lindgren (1955) may accordingly be in agreement with the main part of the experiments in the present study where the vasoconstrictor tone increase was moderate and did not show much of an influence on the vasodilator response.

On the basis of the findings by Falck (1962) (see also Fuxe and Sedvall 1965) that adrenergic fibres reach only the outer part of the arteriolar smooth muscle layer, Folkow (1964 a, b) proposed that the smooth muscles of the arterioles consists of two layers, one internal, myogenically active and one external where the nervous vasoconstrictor activity is exerted. The results by Folkow, Öberg and Rubinstein (1964) that a high prevailing vasconstrictor tone may inhibit the vasodilator response made them suggest that the vasodilator fibres should predominantly innervate the internal sheath of the muscle wall. Since the morphological arrangement of the vasodilator nerves has not been studied it is difficult at present to explain from physiological data the structural inter-relationship between the two types of vasomotor nerves at the peripheral level.

However the present results on unanesthetized dogs indicate that the influence on the vasodilator response exerted by increased vasoconstrictor tone is of minor physiological importance as far as peripheral effects are concerned. Apparently the vasoconstrictor tone does not reach very high levels in the intact conscious dog due to the opposing effects of barostatic mechanisms and a moderate increase in vasoconstrictor tone does not seem to exert any significant antagonism on the effect of vasodilator nerve activity Thus, a sudden demand for increased muscle blood flow can be met by activation of cholinergic vasodilator nerves even if the arterioles are under the influence of sustained constrictor nerve activity Under extreme conditions as during bleeding or circulatory shock, vasodilatation due to activation of cholinergic vasodilator nerves may be inhibited by the prevailing high vasoconstrictor activity

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Reduction of Antral and Duodenal Gastrin Activity by Electrical Vagal Stimulation[1]

By

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Abstract

Fyrö, B. *Reduction of antral and duodenal gastrin activity by electrical vagal stimulation.* Acta physiol. scand. 1967. 71. 334—340.

The effect of electrical vagal stimulation on the antral and duodenal gastrin activity was determined in anesthetized cats. Vagal stimulation for 1 hr at 5, 10 and 20 imp/sec reduced the antral gastrin activity to about 75, 50 and 35 per cent, respectively. Corresponding figures for 2 hrs stimulation was 45, 30 and 25 per cent of the activity in unstimulated cats, thus showing further reduction. Stimulation for 4 hrs did not produce any further reduction. Vagal stimulation for 2 hrs with all 3 frequencies reduced the gastrin activity in the mucosa of the proximal duodenum to unmeasurable levels.

Indirect evidence, demonstrated by means of acid secretion, indicates that vagal impulses cause a release of gastrin from the antral mucosa. Thus, the gastric acid response to vagal activation was reduced or abolished by resection of the antrum (Straaten 1933, Uvnäs 1942, Burstall and Schofield 1954).

A technic developed by Uvnäs and Emås (1961) for assaying the secretory activity of gastrin preparations offered the possibility of making quantitative estimations of antral gastrin activity. In a previous study reserpine was found to reduce the gastrin activity of the antral and duodenal mucosa in cats with intact vagal nerves but not in vagotomized cats (Emås and Fyrö 1965), indicating that vagal innervation was necessary for the reduction induced by reserpine.

The purpose of the present study was to investigate whether vagal stimulation was able to reduce the gastrin activity in antral and duodenal mucosa.

Methods

Experiments were performed on cats (1.8—4.0 kg) fasted for 24 hrs. They were anesthetized with chloralose and urethane (50 + 500 mg/kg b.w. i.v.). The abdominal cavity was opened and a plastic cannula was introduced into the stomach. The cannula was placed oral to the

[1] Part of this investigation were presented at the XII Scandinavian Congress of Physiology in Turku, Finland 1966 (Fyrö 1966).

antrum-corpus boundary to drain off the secretion and to keep the antral mucosa neutral during the experiments. T eliminate regurgitation of intestinal content ligature was tied around the duodenum just proximal to the ampulla of Vater. The animal was then placed on its left side and slight pward tension was exerted on the duodenal ligature to prevent acid from reaching the antral mucosa.

The control cats were anesthetized for the same length of time as the vagally stimulated ones and prepared with a duodenal ligature and gastric cannula.

Stimulation of Vagus Nerves

After insertion of tracheal cannula the vagus nerves were dissected free in the cervical region and cut just below the nodose ganglia. The skin of the neck was folded to form trough, into hich paraffin oil t body temperature was added during the experiment to prevent drying and cooling of the nerves. The peripheral end of each nerv was placed on bipolar silver electrode connected to Grass stimulator (type S 4) delivering monophasic square wave stimuli with duration of 5 msec and an intensity of 4 V. The impulse frequencies were 5, 10 or 20/sec and stimulation was continued for 1, 2 or 4 hrs at each impulse frequency. In all experiments the electrodes were gradually moved peripherally along the nerves to eliminate the effects of local electrode injuries by the stimulation.

Vagal stimulation was performed in 7 series of experiments. In each series, 3 groups of cats (2 cats per group) were stimulated with 5, 10 and 20/sec respectively and 1 group served as control. Vagal stimulation was performed for 1 hr in 3 series, for 2 hrs in 2 series and for 4 hrs in another 2 series of experiments.

Determination of Gastric Acid Secretion

In all experiments the gastric secretion was collected in 15 min periods, the volume was measured and the total acid was determined by titration against 0.01 N NaOH, using phenolphtalein as indicator.

Preparation of Mucosa Specimens

The cats were killed by air embolism and the stomach and proximal duodenum were removed immediately after death. About 2 cm of the antral mucosa were stripped off 0.5 cm proximal to the pyloric sphincter *i.e.* well within the antral area. I one experimental series stimulated for 2 hrs 4 cm segment of the duodenal mucosa was stripped off. The proximal border of the segment was 0.5 cm distal to the pyloric sphincter. The mucus was gently scraped off the specimens and the mucosa was then rapidly frozen and stored —20 C.

Preparation f Gastri

Gastrin was extracted according to the method of Komarov (1938). This method has show satisfactory reproducibility (Emås and Fyrö 1964 and 1965). Gastrin was extracted separately from the pooled mucosa specimens of each group of cats, and simultaneously from the pooled mucosa specimens of all groups within series. Care was taken to treat all batches identically.

Ten extracts were randomly selected and assayed on guinea pig ileum for histamine activity. No histamine activity was detectable in any extract (<0.01 μg of histamine dihydrochloride per mg of dried extract).

Assay of Gastrin

The acid secretory activity of each gastrin extract was assa ed on 4 nonanesthetized gastric fistula cats with histamine as the reference standard as described by Uvnäs and Emås (1961). All extracts from series were assayed on the same 4 animals.

The dose of the antral gastrin extracts was in all assays 0.5 mg/kg and of the extracts from the duodenal mucosa 4 mg/kg. If 4 mg/kg was insufficient to produce response equal to or greater than the response to the small dose of histamine, the response to the extract was recorded as less than that to histamine (Emås and F ro 1964). Such low secretory activity as found only in extracts from the duodenal mucosa of agally stimulated cats.

The secretory activity of the gastrin extracts as expressed in histamine units (HU, Uvnäs and Emås 1961) per mg of dried extract and the antral (duodenal) gastrin activity in HU per g of mucosa. All alues were compared to corresponding control values and expressed in per cent.

Evaluation of Dat

For each series of experiments data ere anal sed in accordance with curren methods for analysis of ariance (Snedecor 1956). I each series one comparison was made between the control group and the 3 stimulated groups aken together.

Results

Gastrin Extracts from the Antral Mucosa

The mean yield of gastrin extract from the control antra was 78.2 ± 4.1 (± standard error of the mean) mg per g frozen mucosa and the mean secretory activity 20.9 ± 5.6 *HU* per mg extract. The mean control value for the antral gastrin activity expressed as HU per g mucosa was 1 660 ± 460.

Effect of vagal stimulation on antral gastrin activity The mean yield of gastrin extract from cats subjected to electrical vagal stimulation was similar to that of the control cats (Fig 1)

The effect of vagal stimulation on the secretory activity of the antral gastrin extracts is given in Fig 2 in per cent of corresponding controls. The mean secretory activity of the extracts from antra of cats stimulated for 1 hr (3 series) at 5 10 and 20 imp sec was 79 48 and 38 per cent. After 2 hrs stimulation (2 series) the gastrin activity was further reduced to 52, 34 and 29 per cent. Following 4 hrs stimulation (2 series) there was no further reduction the secretory activity of the gastrin extracts was 48 39 and 37 per cent of corresponding controls.

For each series of experiments one comparison was made of the activity of the gastrin extracts in the control group to that of the 3 stimulated groups taken together In all series the difference was highly significant ($P<0.001$)

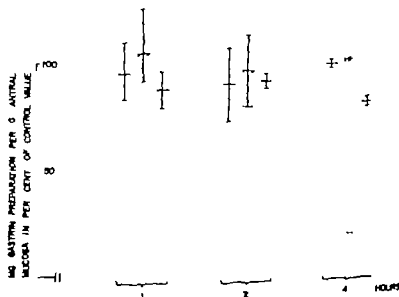


Fig 1 Mean yield of gastrin extract per g antral mucosa of vagally stimulated cats in per cent of corresponding control value Each 1-hr bar represents the mean yield from 3 groups of 2 cats, and each 2 and 4 h bar the mean from 2 groups. The white dotted and dark bars illustrate the effect of 5 10 and 20 imp/sec respectively] = range

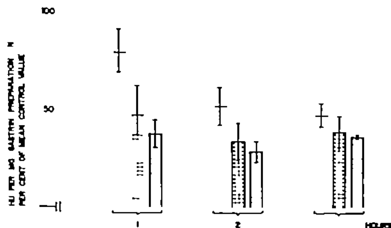


Fig. 2. Mean secretory activity per mg gastrin extract from the antral mucosa of vagally stimulated cats in per cent of corresponding mean control value. Each 1-hr bar represents the mean secretory activity from 3 groups of 2 cats, and each 2- and 4-hr bar the mean from 2 groups. The white, dotted and dark bars illustrate the effect of 5, 10 and 20 imp/sec, respectively. I = range.

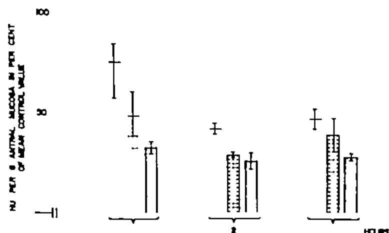


Fig. 3. Mean secretory activity per g antral mucosa of vagally stimulated cats in per cent of corresponding mean control value. Each 1-hr bar represents the mean antral gastrin activity from 3 groups of 2 cats and each 2- and 4-hr bar the mean from 2 groups. The white, dotted and dark bars illustrate the effect of 5, 10 and 20 imp/sec, respectively. I = range.

The antral gastrin activity (HU per g of mucosa, Fig. 3) ran almost parallel to the secretory activity of the gastrin extracts, since the yield of the extracts was fairly uniform and unaltered by vagal stimulation. After stimulation for 1 hr at 5 imp/sec the antral gastrin activity was reduced to 76, at 10 imp/sec to 49, and at 20 imp/sec to 33 per cent of corresponding control values. Stimulation for 2 hrs produced a further decrease of the antral gastrin activity to 43, 29 and 26 per cent. Vagal stimulation for 4 hrs produced no further reduction and the antral gastrin activity amounted to 47, 39 and 28 per cent of corresponding controls.

TABLE I Mean acid output during the consecutive hours in the 4 hr experiments of electrical vagal stimulation. Four cats were stimulated at each frequency

	Mean acid response to electrical vagal stimulation in meq/hr			
Imp/sec	1st hr	2nd hr	3rd hr	4th hr
5	1 41 ± 0.31	2.03 ± 0 47	2.50 ± 0.56	2.15 ± 0.28
10	1 15 ± 0.41	2.17 ± 1 08	1 66 ± 0.50	1.03 ± 0.20
20	1.09 ± 0.25	1 12 ± 0.26	1.33 ± 0 14	1 14 ± 0.22

± S.E.

For each series of experiments there was a highly significant difference ($P < 0.001$) between the antral gastrin activity of the control group and the 3 stimulated groups taken together

Gastrin Extracts from the Duodenal Mucosa

Gastrin was extracted from the duodenal mucosa in one series of experiment following 2 hrs stimulation. The yield of control gastrin extract was 70 mg per g of frozen duodenal mucosa and the mean secretory activity was 1.5 HU per mg extract. The mean control value for duodenal gastrin activity was 104 HU per g mucosa. Microscopic examination of the proximal border of the control duodenal mucosa specimens showed that no antral mucosa was present.

Effect of vagal stimulation on duodenal gastrin activity After stimulation for 2 hrs at 5 10 and 20 imp/sec the yield of gastrin extract was unchanged 97 112 and 111 per cent of control value respectively The mean secretory activity was reduced to < 53 per cent by all 3 frequencies, i.e the activity was reduced below the level measurable by the method. The duodenal gastrin activity (HU per g of mucosa) was calculated to be < 52, < 60 and < 59 per cent of the mean control value

Gastric Acid Secretion

Gastric acid output was recorded in all experiments to test the effectiveness of the vagal stimulation There was no secretion of acid in the control cats whereas electrical vagal stimulation induced acid secretion in all experiments. The mean acid output during the consecutive hours in the 4 hr experiments is given in Table I The antral mucosa was always neutral at the end of the experiments.

Discussion

The release of gastrin by vagal impulses was previously demonstrated indirectly by means of acid secretion. The resection of the gastrin producing area of the stomach reduced or abolished the gastric acid response to electrical vagal stimulation (Uvnäs

1942) and to sham feeding (Straaten 1933 Burstall and Schofield 1954) A sensitive technic allowing quantitative assay of the secretory activity of gastrin preparations has been developed by Uvnäs and Emås (1961) This technic offered the possibility to study experimentally induced changes of the antral gastrin activity The reliability of the methods for assay and extraction (Komarov 1938) of gastrin, which are used in this study have been demonstrated previously (Emås and Fyrö 1964 and 1965)

Experimentally induced changes in antral gastrin activity were first shown by Emås and Fyrö 1965 Thus reserpine was found to reduce the antral gastrin activity in cats to about 40 per cent. A gastrin-like HCl stimulating factor in the proximal duodenum was also found to be reduced by reserpine. After vagotomy however reserpine had no influence on either the antral or the duodenal gastrin activity Therefore, it was suggested that reserpine caused a release of gastrin by central vagal activation.

The present results demonstrate that vagal impulses are able to reduce the antral gastrin activity Earlier reports (Komarov 1938 1942, Harper 1946, Emås and Fyrö 1965) of gastrin-like activity in the proximal duodenal mucosa were confirmed, and vagal stimulation also reduced this activity

The yield of gastrin extract per g antral mucosa was not altered by vagal stimulation. This is to be expected since pure gastrin is only a small fraction of the crude gastrin extracts (*cf* Gregory and Tracy 1964) Thus the reduction in the secretory activity of the gastrin extracts and in the antral (duodenal) gastrin activity (per g mucosa) run almost parallel.

It seems reasonable to ascribe the reduction of the antral and duodenal gastrin activity to a vagally induced release of gastrin. Whether a similar decrease of the gastrin activity occurs under physiological vagal activation remains to be elucidated.

The maximal reduction of antral gastrin activity by electrical vagal stimulation was, in this study about 70 per cent. The same reduction was obtained when the vagal nerves were stimulated for either 2 or 4 hrs. In the 4 hr experiments the acid response to vagal stimulation was roughly the same from the 2nd to the 4th hr demonstrating that the vagal stimulation of the stomach was effective throughout the experiments. The secretory output from the stomach did not decrease during the last 2 hrs of stimulation despite an almost unchanged antral gastrin activity This could indicate that either the vagal stimulation is strong enough to induce secretion without gastrin (Uvnäs *et al* 1966) or that there is a release of gastrin which does not measurably influence the gastrin activity This unchanged gastrin activity suggests either a gastrin pool resistant to vagal impulses and/or continuing gastrin synthesis.

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Acta physiol. scand. 1967. 71. 341—347

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A Micromethod for the Determination of Choline Acetylase in Individual Cells

By

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Abstract

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A micromethod is described for the determination of choline acetylase in single cells. The method involves the use of [^{14}C] acetyl CoA and choline as substrates giving rise to [^{14}C] acetylcholine which is quantitatively precipitated as reineckate. After washing the reineckate to remove the residual radioactivity of the substrate the labelled reineckate is dissolved in acetone and counted in a scintillation counter. The method is suitable for the quantitative determination of enzyme activities in 50—100 individual cells per experiment. The sensitivity of the method is 10^{-14} moles of [^{14}C] acetylcholine/hour.

In a recent communication McCaman and Hunt (1965) described a micromethod for the determination of choline acetylase (ChAc) in nervous tissue. Although this was a considerable improvement over previous methods it was not directly applicable to the determination of ChAc in individual cells. ChAc is accepted as a better indicator of cholinergic function than is cholinesterase so that the determination of ChAc in individual cells is of great importance.

In the present paper the method of McCaman and Hunt is modified and used for the measurement of ChAc in cells from the L7 sympathetic ganglion of the cat.

Methods

Reagent

1) buffer-substrate. Prepared fresh for each experiment to give the final concentrations indicated. 0.1 M phosphate buffer pH 7.4. 0.02 M $MgSO_4$. 1.4 × 10[illegible] M NaCl. 5 × 10[illegible] M

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Abbreviations: acetylcholine = ACh, choline acetylase = ChAc, acetyl co-enzyme A = AcCoA, PPO = 2,5-diphenyloxazole, dimethyl POPOP = 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)-benzene.

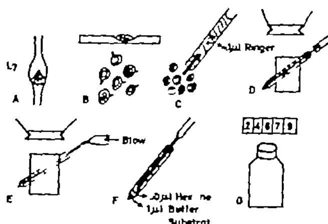


Fig 1 Schematic representation of the procedure for the determination of ChAc in individual cells of the L7 sympathetic ganglion of the cat.
For explanation see text.

choline 2 × 10^{-4} M-eserine 0.05 % bovine albumine 4 7 × 10^{-6} M-[^{14}C]AcCoA (New England Nuclear Corp. 50 mc/m-mole)
ii) choline solution. 5 mM AcCoA (P-L Biochemicals) and 22.5 mM choline in 0.5 N HCl
iii) reinecka e a saturated solution of ammonium reineckate in 0.5 N HCl.
iv) Hyamine 10X as 0.5 M solution in methanol.
) toluene scintillator 5 g PPO 0 1 g dimethyl POPOP per litre of toluene.
vi) detergents. Triton X 100 Nonex 501 as solutions in redistilled water at the concentrations indicated
vii) [^{14}C]Ach, (acetat marked, New England Nuclear Corp. 2.5 mc/m-mole) stored as frozen stock solution in 0 1 M NaH_2PO pH 5.0

A convenient source of enzyme was homogenates of rat brain prepared in a glass homogenizer at concentration of 100 µg/µl redistilled water. Further dilutions of the homogenate ere made with 0 05 % bovine albumen, or with the buffer-substrate. The pipetting of small olumes of the homogenates was facilitated by using the supernatant af er centrifuging at 2,000 rpm for 10 min. The preparation of the homogenates and all manipulations were performed 0- 2 C

L7 ganglia were obtained from anesthetized cats (i.p. Nembutal 30 mg/kg) and placed immediately in isotonic saline or sucrose (buffered to pH 7 4) or in Ringer solution.

After removing the connectiv tissue capsule small piece of tissue was dissected from the middle of the ganglion (F g 1A) and rinsed in several aliquots of the sucrose solution or Ringer. Single cells were obtained from the washed' piece of tissue by microdissection using sharp needles F g 1B) B means of driver micropipette a single cell was washed in fresh Ringer or saline before transferring by the same pipette to small tube (Fig. 1C—1D). The washing of the cell avoided contamination with other material and facilitated the choice of only single cell

The olume of Ringe carried with the cell was approximately 0.1 µl. I some early experiments this was ev porated by blowing (Fig. 1E) but in later experiments this procedure was discontinued

The tubes containing the cells ere placed in ice and 0 5 µl cold buffer-substrate added, followed by 0 5 µl of 0 5 % Triton or Nonex (Fig 1F) Three types ob blanks were investiga ed

i) buffer-substrat w thout tissue
ii) tissue plus buffer-substra thou holine
iii) buffer-substra plus homogenate which had been boiled for 30 min There were no differences in the blank alues obtai ed therefore the blanks used routinely were tubes without tissue

To prevent evaporation the tubes were either sealed with rubber caps or 50 µl hexane was pipetted onto the incubation mixture After incubating for 3 hrs at 38 C the tubes were returned to the ice and the ps or hexane removed. 2 µl of choline solution was added to each tube followed by 2.24 µl of the reineckat solution to precipitat the choline and [^{14}C]Ach The contents of the tubes were then mixed by "buzzing" 7 µl 0.5 N HCl added, and the tubes "buzzed" gain. After tanding in ic for 30 min the tubes were centrifuged in the cold t 3,000 rpm for 15 min. The supernatant containing the [^{14}C]AcCoA was removed and dis-

carded. The precipitate was washed with 16 µl 0.2 N HCl, centrifuged again and the supernatant discarded. The remaining precipitate of [14C]Ach and choline reineckates was dissolved in 31 µl of acetone and transferred with one wash to counting vial. The acetone was allowed to evaporate before adding 0.75 ml 0.3 M Hyamine in methanol followed by 10 ml toluene scintillation counter (Fig. 1G). The complete experimental procedure is show schematically in Fig. 1.

Standards were prepared by adding 1 µl of buffer-substrate directly to the counting vial. Enzyme activities were calculated on the basis of the known specific activity of [14C]acetyl CoA.

Results

Blanks

The optimum conditions for a low blank value and a high recovery of [14C] Ach were chosen after using a range of volumes of choline solution and a range of volumes of reineckate solution. Fig. 2 shows the results of 2 experiments in which the volume of reineckate solution was varied. In the upper curve (10 µl choline solution and 1 µl buffer-substrate) complete precipitation of the choline was not attained until 7 µl of reineckate was added. In the lower curve (2 µl choline solution + 1 µl buffer-substrate) all of the choline was precipitated by the addition of 2 µl reineckate solution. It can be seen that in the upper curve the blank increases as the volume of reineckate is increased, but in the lower curve there is an inverse relationship from the peak at 3 µl reineckate added. These results suggest that the trapping of [14C] AcCoA in the precipitate is proportional to the ratio precipitate/supernatant.

When 7 µl 0.5N HCl is added to the mixture containing excess reineckate the blank is reduced from 859 counts/10 min to 620 counts/10 min without reducing the recovery of [14C] Ach (Table I). A second wash after centrifugation of the precipitate is not so effective in reducing the blank.

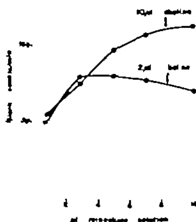


Fig. 2

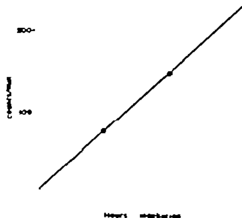


Fig. 3

Fig. 2. The effect on the blank value of increasing volumes of saturated ammonium reineckate solution. 1 µl buffer-substrate plus either µl or 10 µl choline solution. No detergent and wash before centrifugation, all other conditions as methods.

Fig. 3. The production of [14C]Ach by rat brain homogenates (0.25 µg/µl)

TABLE I. Blanks and Recovery of [14C] Ach with and without th ddition of 7 µl, 0.5 NHCl before centrifuging[1]

Blanks ounts/10 min		Recovery of [C] Ach per cent	
without HCl	plus 7 µl 0.5 NHCl	without HCl	plus 7 µl 0.5 NHCl
836	611	97	100
839	624	98	94
887	611	98	97
874	634	93	96
Mean			
859	620	96	97

1 µl buffer-substrate, no detergent, all ther conditions as under methods.

TABLE II The stability and recovery of [14C] Ach

Incubation time (hrs)	Temp. C	[14C] Ach Incubated with —	Recovery of [14C] Ach per cen
0	—	Buffer pH 7 4	94
1	38	Buffer pH 7 4	96
2	38	Buffer pH 7 4	98
3	38	Buffer pH 7 4	98
1	38	Homogenate + Eserine 2 10⁻⁵M	96
3	38	Triton 0.1	95
3	38	Tri on 0.3	95
3	38	Triton 0.5	95

Stability of A h

The stability of added [4C] Ach was determined after incubation for arious times both with and without tissue Table II hows that [14C] Ach was table for at least 3 hrs at 38 C and was not h drolysed by brain ChEs in the presence of 2 10 5M eserine Triton X 100 did not affect the recovery up to a concentration of 0.5 %

The identity of the reineckate precipitate as [14C] Ach was indicated by experiments using homogenates These showed that without choline there was no enzyme activity and without eserine th yield was only 30 per cent.

1 The blank values reported in this paper has not been corrected for background. The background alue was about 400 ount 10 min

TABLE III. Th relationship between tissue concentration nd Ach production

Experiment 1		Experiment 2	
µg brain/µl	counts/10 min (mean of 3)	µg brain/µl	cou ts/10 min (M ± S.D., N = 3
0.01	0	0.07	81 ± 20
0.1	167	0.7	680 ± 120
1.0	1490	7 0	6 790 ± 887

1 µl of buffer-substrate containing homogenate, incubated for 1 hr t 38° C. N detergent. All other conditions as described under methods.

TABLE IV Choline acetylase in individual cells of the L7 ganglion of the cat

Blanks		Cells	
counts/10 min.		counts/10 min.	
667	539	564	581
513	627	602	578
551	671	549	568
575	564	569	788
593	528	644	780
579	566	673	570
629	542	403	614
512	562	585	570
637	594	582	591
602	585	579	595
585 ± 48	(M ± S.D)	active cells marked	

Standard 4.9 10 moles [^{14}C] AcCoA = 24 100 counts 10 min. Sensitivity limit 1 9 10 moles [^{14}C] Ach. The lues for be bla ks and the cells reported in th table belong to the same experiment.

Th relationship between n bation t m nzym nc nt ation and th synth sis of Ach

The relationship betwen the production of Ach and enzyme concentration was n-vestigated over range of tissue concentrations. The results, shown in Table III suggest that over at least h ndred-fold range the ield of Ach is proportional t the quantity of enzyme. Using small amounts of brain tissue it was established that fter 3 hrs incubation the observed activity was 90 per cent of that at 1 hr (Fig 3 When the buffer-substrate and homogenate were incubated separately for 3 hrs at 38 C, the activity measured in a subsequent incubation for 1 hr was the same as that i controls w thout the pre-incubation.

A typical experiment with cells from the L7 sympathetic ganglion of the cat is shown in Table IV. Cells were considered active when the counts were above the mean blank by more than $3.62 \times$ S.D. of the blanks (see discussion). The sensitivity limit of the method is therefore governed by the value $3.62 \times$ S.D. of the blanks. In most experiments the lowest detectable ChAc activity was of the order of 10^{-13} moles Ach.

In the experiment illustrated two of the 20 cells were active forming 4 and 4.1×10^{-13} moles of [^{14}C] Ach per cell per hour.

Discussion

McCaman and Hunt (1965) showed that the activity of brain after 120 min incubation was 75 per cent of that obtained at 60 min. They could not decide whether this was due to instability of the enzyme or to hydrolysis of the AcCoA which is the limiting substrate. The present experiments suggest that the AcCoA is limiting since with small amounts of brain the activity at 3 hrs was only reduced 10 per cent compared to the activity at 1 hr. Furthermore pre-incubation of the enzyme and substrate separately has no effect on the activity. The results suggest that with amounts of enzyme present in single cells the production of Ach is proportional to time and enzyme for at least 3 hrs.

The recovery of Ach is quantitative and not affected by the presence of detergents (Table II) at the concentrations used. Added [^{14}C] Ach is stable in the buffer-substrate and is not hydrolysed by brain homogenates in the presence of 2×10^{-4}M eserine. Thus any [^{14}C] Ach formed by the cells will be recovered quantitatively at the end of the incubation.

Fonnum (1966) demonstrated activation of ChAc in brain homogenates by the use of Nonex 501 and Triton X 100 at concentrations of 0.5 %. This was confirmed using homogenates prepared at a concentration of 100 μg tissue/μl, but when the detergent was added to very dilute homogenates (1—2 μg/μl) the activation was only 10 per cent instead of 100 per cent. When the activities of cells were compared in the presence and absence of 0.25 % Triton or of 0.25 % Nonex no difference was found in the percentage of active cells or in the range of activity observed. Fonnum (1967) has shown that ChAc can be obtained in either a bound or soluble form depending on the ionic strength and pH. The incubation of cells in the high ionic strength buffer substrate should leave the ChAc in the soluble phase so that detergents would not then produce further activation. This remains to be confirmed.

In the L7 ganglion most of the cells were not active. The possibility that only 10 per cent of the cells reached the incubation mixture was examined. When cells stained with methylene blue were pipetted in the manner described under methods only one of 50 cells was not visible in the 1 μl of Ringer added subsequently.

I normally distributed population 95 per cent of the observations will lie within the range mean $\pm 2 \times$ S.D. Saunders and Fleming 1957.
The upper "tolerance limit" of normal distribution can be calculated from sample of observations according to the formula

$$\text{mean} + k \times \text{S.D.}$$

here k is constant obtained from "Documenta Geigy Scientific Tables With tolerance probability of 99 per cent, confidence probability of 95 per cent and = 20 (Table IV) the constant k is 3 62. I this case at least 99.5 per cent of the distribution is less than the tolerance limit.

When a distribution curve is constructed for the blanks and cell counts it was seen that the cells contained two populations. The majority of the cells gave counts within the range of the blanks but some cells were outside this range (Table IV) These latter cells were also outside the range 3 6? $\times$ S.D of the blanks (Table IV) It was concluded that these cells were active and the remainder were either inactive or possessed ChAc activity lower than 10^{-5} moles of Ach per hour

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Cholinacetylase in Innervated and Denervated Sympathetic Ganglia and Ganglion Cells of the Cat

By

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Abstract

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Cholinacetylase (ChA) activity was determined in normal and denervated lumbosacral sympathetic ganglia of the cat. Preganglionic denervation resulted in 98 per cent decrease of the ChAc. The postsynaptic ChAc activity was related t the proportion of presumably cholinergic cell bodies in the denervated ganglion. ChA was found in about 13 per cent of the cell bodies in the 7th lumbar ganglion before as well as fter denervation The average enzyme activity in the cell decreased by about 50 per cent after denervation. The results indicate that ChAc is rather specifically located in cholinergic presynaptic and postsynaptic structures. The data favour the concept that sympathetic ganglia of the cat contain t least two different cell populations with distinct cytochemical, histochemical and pharmacological haracteristics.

Pharmacological, biochemical and histochemical data support the concept that the sympathetic ganglia cell population is functionally heterogenous.

It has been suggested that two main cell types the cholinergic and the adrenergic, can be distinguished by various histochemical and biochemical techniques. The former are characterized by heavy staining for acetylcholinesterase (AcChE) (Sjöqvist 1962, Fredricsson and Sjöqvist 1962, Sjöqvist 1963) which has been shown to reflect a high acti ty of this enzyme (Holmstedt *et al* 1963 Giacobini *et al.* 1967) These cells do not contain monoamines in contrast to the majority which exhibit various degrees of fluorescence for noradrenaline (Hamberger *et al* 1963, 1965) Histochemical (Sjöqvist 1962, 1963) as well as cytochemical studies (Giacobini *et al.* 1967) have shown that less than 13 per cent of the cell bodies in the 7th lumbar ganglion, have high AcChE activ ty while the remainder show only traces of activity or no activity at all

It has been questioned (Sjöqvist 1962, Holmstedt *et al* 1963 Giacobini *et al.*

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1967) whether low AcChE activity in ganglionic cell bodies really reflects the participation of acetylcholine (ACh) in transmission in the corresponding nerve terminals, as suggested by Koelle (1962, 1966) Burn and Rand (1962) and Eränkö (1966)

The ACh synthesizing enzyme, cholinacetylase (ChAc) seems to be more specifically localized in cholinergic neurons (Hebb 1962 Hebb and Krnjevic 1962) Parallel studies of AcChE and ChAc have so far been carried out on relatively large areas of the nervous system and have given little information about the cellular distribution of the two enzymes (Hebb 1962, Hebb and Krnjevic 1962) We therefore decided to study the localization of ChAc in the 7th lumbar ganglion of the cat with the main object of establishing the proportion of ganglion cells containing this enzyme. This study was carried out using a recently developed micromethod for individual cells (McCaman and Hunt 1965 Buckley *et al.* 1967) In addition, the effect of preganglionic denervation on the ChAc levels in various sympathetic ganglia was investigated.

Material and methods

A. *Dissection and denervation procedure*

The experiments were performed on sympathetic ganglia of adult cats of both sexes. The animals were anesthetized with sodium pentobarbital (Nembutal, Abbott, 40 mg/kg, i.p.) The lumbosacral ganglia were dissected free and either removed for immediate biochemical analysis or denervated as follows. The sympathetic chain was removed bilaterally between L3 and L5 for a length of about 2 cm. The sympathetic trunk was then cut between L5 and L6 The operation was performed under aseptic conditions as previously described (Holmstedt, Lundgren and Sjöqvist 1963) There was no bleeding during the operations and no significant tissue reactions were found around the sympathetic chain at the subsequent dissection of the ganglia 2—7 weeks later The analysis of the ChAc content of the denervated ganglia revealed, somewhat surprisingly, that some regeneration of the enzyme activity must have occurred at survival times exceeding 4 weeks (*vide infra*) The recovery of the activity decreased in a distal direction from L6 to L7 and S1 respectively Table II The operative procedure was therefore modified to include a complete resection of L5 as well It should be pointed out that previous data indicate that few if any preganglionic fibres enter the chain below L4 and probably none below the level of L5 (Langley 1892 Holmstedt *et al.* 1963 Some cats later the subsequent dissection, there was no microscopical sign of regeneration in these cats. However in a few cats the resection of the chain had been misplaced and included L6 This fact together with anatomical irregularities in the occurrence of the different ganglia explain the differences in the number of ganglia investigated (Table II

The dissected ganglia were either weighed, homogenized and analyzed for total ChAc activity or placed in saline for preparation of individual ganglion cells under the dissection microscope. In the latter case individual cell bodies were quickly isolated by means of gentle manipulation using sharp needles and sucked into a Carlsberg diver micropipette Giacobini 1957) The cells were then transferred to a glass tube according to the technique described elsewhere (Buckley *et al.* 1967) Each preparation included one individual cell body together with the initial segment of the axon, approximately 50 μ long (Giacobini 1957 The volume of Ringer or saline carried with the cells was approximately 0.1 μl The presence of the cell preparation in the bottom of the tube was verified under the microscope After the addition of the buffer-substrate or buffer-substrate-detergent solution the histological features of the cell bodies were no longer discernible

B *Biochemical and microchemical determinations*

Cholinacetylase activity was determined by the method of McCaman and Hunt (1965) using (^{14}C) acetyl CoA (New England Nuclear Corp. 50 mC/mmole This method has modified by Buckley *et al.* 1967) to measure the enzymatic activity of isolated cells suitable for the determination of enzyme activities in up to 40 individual cell per ex

TABLE I Cholinacetylase in various autonomic ganglia of the cat

Ganglion	S_1	L_7	L_6	Stellate	Coeliac	Superior cervical	Ciliary
ChAc[1]	100 ± 0.9	100 ± 0.5	88 ± 0.9	120 ± 10.6	48 ± 3.2	173 ± 6.6	242 ± 18
	(5)	(5)	(5)	(4)	(3)	(6)	(3)
AcChE	8.42 ± 0.54	8.70 ± 0 78	5.53 ± 0.22	8.91 ± 0.97	4 46 ± 0.41	9.21 ± 0.30	15[3]
	(7)	(10)	(10)	(4)	(8)	(4)	(3)

ChAc activity expressed [illegible] moles ACh × 10 [illegible] /hour/μg wet. The values represent means ± S.E. Number of cats within parenthesis.

Holmstedt, B., G Lundgren and F Sjöqvist, Acta physiol. scand. 1963 57 235—247 AcChE activity expressed in μmoles acid × 10⁶ mg⁻¹/min The values represent means ± S.E.

Sjöqvist, F 1962

The (^{14}C ACh) produced was measured in Packard liquid scintillation spectrometer When distribution curve was constructed for the blanks and cell counts it was seen that the cells belonged to two separate populations. The majority of the cells gave counts within the range of the blanks but some cells were outside this range. These latter cells were also outside the range mean + 3.62 × S.D of the blanks (= 20 tolerance probability of 99 per cent and confidence probability of 25 per cent, K = 3.62 according to Buckley et al. 1967)

Thus, cells were only considered active when the counts exceeded the mean blank by more than 3 62 × S D The sensitivity limit of the method is therefore governed by the size 3.62 × S.D of the blanks I most experiments the lowest detectable ChAc activity was of the order of 10^{-13} moles ACh/cell/hour but few cells showed an activity of the order of 0 5 × 10^{-13} moles ACh/cell/hour

Results

The ChAc activities of different normal ganglia are shown in Table I Among the paravertebral ganglia, the stellate L7 and S1 show a high level of activity and the coeliac a rather low activity When comparing the ChAc activities with the quoted AcChE data one finds a good agreement between the two. For comparison a few determinations of ChAc and AcChE activities in the ciliary ganglion have been included Both enzymes have considerably higher activity in this parasympathetic ganglion than in the sympathetic ones.

Table II shows the ChAc values in the lumbosacral ganglia at different times after denervation. Two weeks thereafter there was a 98 per cent decrease in ChAc activity in the three sympathetic ganglia investigated After a further survival of 1—2 weeks the ChAc level is slightly higher but still very low The 7th lumbar ganglion has the highest postsynaptic ChAc activity followed by S1 and L6. This is surprisingly well correlated to the number of presumed cholinergic perikarya in these ganglia, which constitute 12.8, 9 7 and 5 3 per cent respectively of the entire population of cells (Sjöqvist 1962 1963) Using the first procedure for denervation as described under Methods it was found that the ChAc activity had regenerated 6—7 weeks after denervation and L6 then had enzyme activity as high as L7 and

TABLE II Cholinacetylase in lumbosacral ganglia t different time periods after preganglionic denervation

Experimental procedure	Ganglion		
	L_6	L_7	S_1
Normal	88 ± 0.9 (5)	100 ± 0.5 (5)	100 ± 6 9 (5)
2 weeks after denervation	1 6 ± 0.2 (5)	2.2 ± 0.2 (7)	2.2 ± 0.2 (7)
3—4 weeks after deners tion	2.0 ± 0.2 (9)	3.0 ± 0.2 (11)	2.6 ± 0.4 (8)
6—7 weeks after denervation	15 ± 2 2 (5)	14 ± 1.8 (6)	11 ± 1 4 (7)

[1]ChAc activity (means ± S.E.) expressed in moles Ach 10^{-11}/hour/µg wet (umber of cats within parenthesis).

These data are not comparable with the others bec use of the change in denervation procedur described under Material and Methods

TABLE III Per cent decrease in ChAc and AcChE activ ties in sympathetic ganglia following denervation

Ganglion	Decrease in ChAc activ ty 2 weeks after denerv tion (in per cent of control	Decrease in AcChE activity 1—3 weeks after denervation (in per cent of control)
L6	98.2	64
L7	97 8	48
S1	97.8	53

Sjöqvist, F 1962.

even higher activity than S1 This probably reflects reinnervation of the ganglia, taking place from the peroneal direction, caused by incomplete denervation.

Preganglionic denervation results n loss of both ChAc and AChE activit in the ganglia (Table III) The decrease enzyme activity is about 98 per cent in all ganglia for ChAc but only between 48 and 64 per cent for AcChE, ndica ing that the former enzyme is more specificall located at the presynaptic level

The material reported i Table IV nd b includes several experiment per formed in 405 isolated cell bodies from the 7th lumbar ganglion. 216 cells were dissected from seven normal ganglia and 189 from three denervated ganglia The 27 normal cells w th measurable ChA show ctivities from 13.6 to 0.8 10 moles ACh/hou These cells represent 1 5 per cent of the investigated population Cell bodies w th low ChA acti ity represent the majority of the population, hile those w th ery high acti ty are rare

TABLE IV. A. ChAc activity (in moles ACh × 10 [illegible]/hour/cell) in normal and denervated sympathetic ganglion cells of the cat 7th lumbar ganglion

Normal ganglion cells		Denervated ganglion cells	
	1.5		
13.6	1.3	3.0	1.3
11.8	1.3	2.2	1.3
4.3	1.3	1.9	1.2
4.1	1.3	1.9	0.7
4.0	1.3	1.8	0.6
3 7	1.3	1.8	0.6
2.5	1.2	1.8	0.6
1 8	1 1	1 7	0.6
1 8	1 1	1 6	0.5
1 7	1 0	1.5	0.5
1 7	1 0	1.5	0.5
1 6	0.9	1.3	0.5
1.5	0.8		0.5
Active cells	n = 27		= 25
	M_t = 2 61 ± 0.60		M = 1.2 3 ± 0.13
Inactive cells	n = 189		= 164
Total cells	n = 216		n = 189
Per cent active cells	12.5		13.2

TABLE IV. B. Distribution of cells with and without cholinacetylase [illegible]ctivity in different experiments from normal and denervated 7th lumbar ganglia

Exp. no	Acti[illegible]	Inactive	Total	Per cent [illegible]ctive
Normal ganglion cells				
I	5	35	40	12.5
II	5	34	39	12.8
III	3	27	30	10 0
IV	3	17	20	15.0
V	5	10	15	33 0
VI	4	43	47	8.5
VII	2	23	25	8.0
7	27 (12 5 [illegible])	189	216	M_t = 14 2
Denervated ganglion cells				
I	9	61	70	12.8
II	7	73	80	8.8
III	9	30	39	23.0
n 3	25 (13.2 [illegible])	164	189	M_t = 14 8

After denervation the proportion of active cells remained approximately the same (13.2 per cent). These figures agree well with the previous finding of 12.8 per cent of AcChE-stained, presumably cholinergic, cell bodies in the 7th lumbar ganglion of the cat (Sjöqvist 1962, 1963). The difference between the individual values becomes less after denervation and a decrease in the average enzyme activity from 2.6 to 1.25×10^{-7} moles ACh/hour occurs as well.

Discussion

Autonomic ganglia with their preganglionic nerve trunks and motor spinal roots have the highest concentrations of ACh, AcChE and ChAc in the peripheral nervous system (*cf.* Hebb and Krnjević 1965). Our data show a good agreement between the activities of AcChE and ChAc in autonomic ganglia. The highest values of the two enzymes were found in the ciliary and superior cervical ganglia and the lowest in the coeliac ganglion. The denervation experiments (*vide infra*) revealed that most of the ChAc is located presynaptically. The differences in ChAc activity between various ganglia therefore probably reflect corresponding differences in the density and arborization of the preganglionic terminals.

Two to four weeks after preganglionic denervation the ChAc activity in the three lumbosacral ganglia investigated had dropped to less than 3 per cent of the control. By contrast, the AcChE activity in the corresponding ganglia remained as high as 36 to 52 per cent of the control. These data show that ChAc is much more specifically located at the sympathetic presynaptic sites.

Preganglionic denervation results in morphological changes in the presynaptic terminals even within 24 hrs. They consist of swelling of mitochondria and clumping of vesicles (Hunt and Nelson 1965). By contrast no obvious morphological changes occur in the postsynaptic elements (*loc. cit.*). To judge from histochemical studies (Koelle 1951, Fredricsson and Sjöqvist 1962, Holmstedt *et al.* 1963) preganglionic denervation does not affect the AcChE activity of the cell bodies. The fact that only minute ChAc activities remained in the sympathetic ganglia after denervation was therefore taken as evidence for a rather restricted postsynaptic distribution of this enzyme as compared to AcChE. Further support for this view was obtained when comparing the ChAc activities of the denervated L6, L7 and S1 ganglia. The ChAc activity was roughly correlated to the proportion of presumed cholinergic cell bodies in the respective ganglion (*cf.* Results). This indicated that the ChAc occurred more or less specifically in the cholinergic perikarya.

Direct evidence for this view was obtained in experiments with isolated cells. ChAc could be demonstrated in only about 13 per cent of the cell bodies in the 7th lumbar ganglion. [illegible] figure similar to the proportion of ganglion cells which has previously been suggested to be cholinergic on the basis of correlated histochemical (AcChE) and pharmacological experiments (Sjöqvist 1962, 1963 a, b).

Thus, a limited proportion of the cell bodies in the 7th lumbar ganglion contains the enzyme required for the synthesis of acetylcholine. Experiments are in progress

to determine whether these cell bodies store the transmitter or not. It is known that the adrenergic sympathetic cell bodies contain noradrenaline (Falck 1962, Hamberger *et al.* 1963 1965) and apparently this transmitter is in transit from the perikarya to the axonal terminals (Dahlström 1965) The same situation may exist in the cholinergic neurons.

Our experiments showed that the percentage of cells with ChAc activity remained the same after denervation, although the average enzyme activity decreased by more than 50 per cent. This could mean that the preganglionically located enzyme is especially concentrated in those terminals which abut on the cholinergic perikarya. It is known that preparations of isolated cells contain synaptic terminals as well. Alternatively a postsynaptic decrease in the activity had been induced. The results may also be interpreted to indicate that the largest part of ChAc is not attached to the synaptic boutons but localized more proximally in preterminal axons. This would differ from the localization of AcChE (Giacobini *et al.* 1967) which is distributed in the synaptic boutons of all cells. Axonal ChAc must then either be derived from the cell body (see above) or be synthesized locally In the latter case, synthesis may take place in the axonal part proximal to the terminals or in surrounding cells (glial cells, Schwann cells) The ability of the axon to synthesize enzymes (Koenig 1965) and the presence of messenger RNA in it (Edström *et al.* 1962) make the former explanation more likely

The cell population in the 7th lumbar ganglion of the cat has turned out to be useful for correlated functional and cytochemical studies. From this ganglion originate both cholinergic (sweat secretory and vasodilator) and adrenergic (vasoconstrictor) fibres (Sjöqvist 1963a)

The scheme in Fig. 1 shows that the number of ganglion cells in L7 which contain AcChE (without any distinction between those with high and those with low activity) considerably exceeds the proportion of cells lacking monoamines but containing ChAc This illustrates the fact that AcChE occurs in adrenergic cell bodies as well (*cf* Giacobini *et al* 1967) It is questionable whether a low AcChE activity

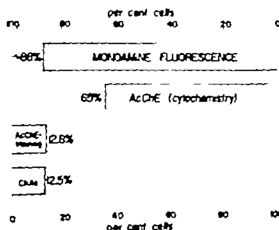


Fig. 1 Diagram of the percentage of the cells from the 7th lumbar ganglion of the cat containing AcChE, ChAc and monoamines.

in a neuron can be considered as evidence for a cholinergic function (*cf* Fredricsson and Sjöqvist 1962, Holmstedt *et al.* 1963 Giacobini *et al* 1967) In support of this view Goldberg and McCaman (1967) found little or no correlation between the relative levels of ChAc and AcChE in the various cerebellar layers. It is likely that the ChAc rather than the AcChE activity of a cell body reflects the participation of ACh in the transmission process in the corresponding nerve terminals.

Accepting this view it seems quite likely that the sympathetic ganglia of the cat contain at least two distinct cell populations. The cholinergic, representing around 15 per cent of the ganglion cells, is characterized by hea y histochemical staining for AcChE, high AcChE activity as determined cytochemically high ChAc activity and the absence of monoamines (Fig 1) The adrenergic exhibits fluorescence for monoamines (noradrenaline) contains traces of AcChE or no activity at all, but has no measurable ChAc activity There is no reason to believe that noradrenaline plays a role in the former neurons. The absence of measurable levels of ChAc in presumably adrenergic cell bodies does not fa ou the concept of a cholinergic link in adrenergic transmission. However negative biochemical findings must be interpreted with caution, until even more sensitive methods ha e been developed.

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Histamine Release from Rat Mast Cell Granules Induced by Bee Venom Fractions

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Abstract

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Mast cell granules, obtained from sonically disintegrated rat peritoneal cells and suspended in sucrose 0.34 M, were found to release histamine in the presence of bee venom. Three venom fractions were obtained by gel filtration, two of these had strong histamine releasing capacity, namely "F I" containing phosphatidase A, and "F III" with direct hemolytic properties. The third fraction, "F II", which releases histamine from intact mast cells, had little effect on granules. Various possibilities for the release mechanism, namely phosphatidase activity, surface activity, and cation exchange, are discussed.

Most, perhaps all, of the histamine in mast cells is stored in intracellular granules. Such granules have been prepared from rat peritoneal mast cells by sonic disintegration (Lagunoff and Benditt 1963, Lagunoff *et al.* 1964) or by hypoosmotic lysis of the cells (Uvnäs 1964). Recent studies have given strong support to the idea that the histamine is bound electrostatically to the heparin-protein complex constituting the granular stroma. This view is based on the finding that simple cations, e.g. Na and K, are able to release histamine from isolated granules (Lagunoff *et al.* 1964, Uvnäs 1964, Thon and Uvnäs 1966) probably by ion exchange as corresponding amount of cations is taken up by the granules (Åborg, Novotny and Uvnäs 1967). It has been pointed out that in addition to electrostatic forces some other factors, e.g. hydrogen bonding and Donnan equilibria, may be involved in the binding of histamine (Green 1966).

Histamine release from intact mast cells induced by some histamine liberating agents, e.g. antigen and compound 48/80, is due to activation of a cellular energy requiring mechanism (for ref. see Uvnäs 1961). It has been found that these agents do not release histamine from isolated granules in the low concentrations that are sufficient to release histamine from intact cells (Mongar and Schild 1954, Uvnäs 1964). However, in a preliminary study it was found that bee venom prepar-

— which was assumed to act on whole cells in a way similar to compound 48/80 (Högberg and Uvnäs 1960) — also released histamine from isolated granules (Haegermark, unpublished). Later it was found that this venom preparation could be separated into two fractions, one containing phosphatidase A (Fraction I) and one with mast cell degranulating properties (Fraction II) (Fredholm 1966). The aim of the present work was to find out if the histamine release from granules was induced by another fraction than that affecting intact mast cells.

Methods

Fractionation of bee venom

The bee venom preparation was fractionated by gel filtration on Sephadex G 50 as described previously (Fredholm 1966, Fredholm and Haegermark 1967). The column measured 24 × 520 mm, and it was equilibrated and eluted with 0.01 M NH_4HCO_3, pH 7.9. Fiv mg of bee venom was used in each run, and twenty 12-ml fractions were collected. The fractions were screened for phosphatidase activity and for *histamine releasing effect on intact mast cells*. Fractions 7—9, containing phosphatidase A, were pooled and freeze-dried and termed F I. Fractions 15—20, causing histamine release from mast cells, were treated similarly and termed F II. The fractions thus obtained corresponded to the F I and F II described by Fredholm and Haegermark (1967). During the progress of the investigation it was found that some of the material eluted between F I and F II had marked *histamine releasing effect on mast* cell granules as well as on intact mast cells. This (tubes 12—14) was referred to as F III and was included the study.

Electrophoresis

Low voltage paper electrophoresis was performed on LKB 3290 B apparatus using 40 mm wide strips of Whatman No. 1 paper. The runs were carried out in 0.1 M glycine—NaOH buffer. The current was 0.3—0.6 mA/cm and running time 20 hours. After drying, a longitudinally cut strip was stained with amido black, and the rest of the paper was cut transversally into 1-cm pieces, which were eluted with 0.01 M NH_4HCO_3.

Preparation of granules

The technique for preparation of granules was modification of that described by Lagunoff *et al.* 1963, 1964). Peritoneal cells from male Sprague-Dawley rats (400—300 g) were collected as described previously Fredholm and Haegermark 1967). Usually 2—3 animals were used in each experiment and the cells were pooled. To remove extracellular cations the cells were washed once in ice-cold sucrose solution (0.34 M) adjusted to pH 7 with NaOH, 0.02—0.04 mmole/l. All preparatory steps hereafter were undertaken at 0° C. After re-suspension in 15 ml of fresh sucrose solution the cells were disintegrated by sonic vibration at 10 kHz for 30 sec in Raytheon sonicator operating at 1 A output. Microscopic examination after this procedure usually revealed no tac lls. The suspension was then centrifuged at 350 × g for 5 min to remove whole or partially disrupted cells (this sediment also contained some large granules. The granules were then sedimented by centrifugation at 1,500 × g for 45 min, resuspended in sucrose solution and used for incubation.

The optimal conditions for the disintegration procedure had previously been established by treating 15 ml aliquots of mast cell suspension the sonicator for various periods of time (Fig. 1). At time 0 no sonication, all the histamine was recovered in the cell fraction (350 × g sediment). In suspensions treated for 15—30 sec the granular fraction contained 85 per cent of the histamine. Further prolongation of the sonication time resulted in decrease of granular histamine.

In the electron microscope was observed that the granule suspension used for incubation contained mostly mast cell granules, although not more than about 5 per cent of the peritoneal cells are mast cells. A few eosinophilic granules and a few mitochondria were seen (Bloom and Haegermark unpublished).

In some experiments, mast cells were separated from the other peritoneal cells by density gradient centrifugation in Ficoll (Lunas and Thon 1959, Thon and Uvnäs 1966). Granules were prepared from these cells according the scheme described above.

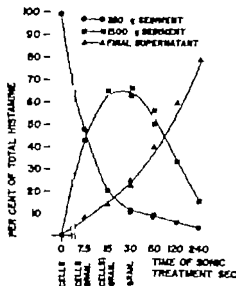


Fig 1 Effect of sonication time on histamine distribution. Histamine was determined in cell fraction (330 × g sediment) "granule fraction (1,500 g sediment), and final supernatant after sonic treatment of cell suspensions. Tw expts, each point represents the mean of duplicate determinations. The microscopic ppearance of the treated samples is denoted below the diagram.

Incubation t hnique

The incubations were performed in 15 ml plastic centrifuge tubes. A 0.1 ml sample of the liberator solution was introduced into each tube followed by 0.9 ml of the granule suspension. The tubes were incubated at 37 C for 10 min unless otherwise stated. At the end of the incubation the tubes were placed in iced water and immediately centrifugd 1,500 × g for 45 min. Half millilitre of the supernatant was carefully removed with pipette. The histamine that remained in the granules was extracted by adding 5.5 ml saline to the sediment tube (sediment + 0.5 ml supernatant) and heating for 3 min at 100 C. Histamine was determined in both supernatant and sediment and the release was calculated as the percentage of the total amount — usually —4 µg — in each tube. All figures given are based on results from duplicate samples.

That the histamine recovered in the supernatant was actually released and not particle-bound was supported by the finding that ultracentrifugation (200 000 × g for 60 min) of the supernatant failed to sediment the released histamine

Histamine assay

Histamine was measured by fluorometry fter condensation with o-phthalaldehyde (OPT) according to Shore, Burkhalter and Cohn (1959). The purification steps were omitted, however as no substances interfering th the histamine assay were present, and OPT was added directly to the alkalinized samples

Measurement of h parin elease

Sulphate is formed to be incorporated int mast ell heparin (Korn 1959 Lagunoff Calhoun and Benditt 1960) T obtain mast cells ith S^{35} labelled heparin, rats were injected with 2 ml of $Na_2S^{35}O_4$, 1 mCi/ml. T days later the peritoneal cells ere collected from the rats and the mast cells were isolated in Ficoll. Granules containing S^{35}-heparin were prepared from these mast cells and incubated th liberator. Heparin release was estimated by measuring radioactivity in the supernatant and he sediment, using Tri-Carb liquid scintillation spectrometer (Packard Instrumen Co

Assay of phosphatidase A activity

The phosphatidase A activ ty was determined by measuring the decrease in acyl ester bonds after hydrolysis of lecithin as described previously Fredholm 1966) The reaction was allowed to proceed for 10 min and the amount of intact acyl ester bonds was assayed col— according to Stern and Shapiro (1953

Measurement of surface tension

Surface tension at the air liquid interface was determined with a platinum-iridium ring and torsion balance (Verenigde Draadfabrieken, Nijmegen, The Netherlands). The pull necessary to detach the ring from the surface was registered and surface tension, expressed as dyn/cm, was calculated according to Harkins and Jordan (1930).

Materials

A bee venom preparation (in the following called "bee venom") obtained by precipitation with picric acid and chromatography on Amberlite IRC-50 was generously supplied by Dr B. Högberg, AB Leo, Hälsingborg, Sweden.

Lecithin (egg) was prepared and kindly supplied by Professor A. Wretlind, National Institute of Public Health, Stockholm, Sweden.

$Na_2S^{35}O_4$ was obtained from Institutt for Atomenergi, Kjeller, Norway.

Results

Histamine release by different bee venom fractions

The releasing capacity on a weight basis, was about the same for unfractionated bee venom, the phosphatidase A containing fraction (F I) and the fraction F III 1 µg/ml inducing 25—40 per cent release. The mast cell degranulating fraction (F II) did not cause any release from the granules even when present at 10 µg/ml. A still higher concentration of F II 50 µg/ml, induced histamine release (Fig. 2a).

The effect of the different fractions was also tested on a purer granule suspension, obtained by disintegration of isolated mast cells. The results, shown in Fig. 2b were very similar to those with granules from unfractionated cells.

Time course of the histamine release

Since the release process induced by bee venom and its fractions was found to be only moderately decreased at 0 C (see below) and the centrifugation time after incubation was as long as 45 min, the usual handling of the samples would not lead to an immediate arrest of the histamine release at the end of the incubation. Consequently this technique was not well suited to study the time course of the reaction. Therefore

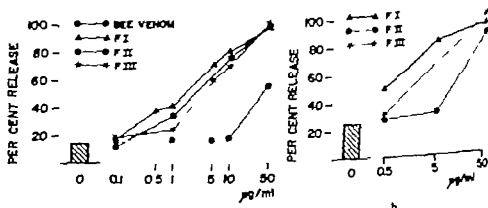


Fig. 2. Concentration-response curves for histamine release induced by bee venom and the bee venom fractions. The granules were incubated at 37° C for 10 min. a) Granules from unfractionated cells. Each point represents the mean value of 3—5 expts. b) Granules from isolated cells. Each point is the mean of duplicate determination.

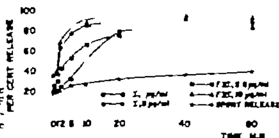


Fig. 3. Time course of histamine release induced by different concentrations of F I and F III at 37° C. Each curve represents one expt. The spontaneous release indicated is the mean of the four expts.

the incubation was instead terminated after various periods of time by adding 9 ml of ice-cold sucrose to the tubes, thereby lowering the liberator concentration tenfold. This treatment was considered to result in a more rapid termination of the release process.

The initial rate of release was found to increase with increasing concentration of the liberating agent, but, independent of the concentration, the final release level was reached after about 20 min. No significant difference was found between th different releasing fractions (Fig. 3)

Histamine releasing and lecithin splitti g act vities of F I

Fraction F I contains the principal part of the phosphatidase A activity of th bee venom. This fact suggested the hypothesis that the histamine release could be ascribed to this enzyme. Supporting this idea was the f nding that the histamine releasing and phosphatidase A activities were recovered in the same area on paper electrophoresis with this technique the isoelectric point for both was found to be around pH 11.5. Additional evidence was obtained from the fact that a highl puri fied phosphatidase A prepared in a different way exhibited a histamine releasing effect corresponding to that of our preparation. In the following paragraphs some experiments are described, where histamine release and lecithin hydrolysis were compared. Due consideration must be paid to the fact that the two reactions were not run under identical cond tions.

Selective heat inactivation f th ven m and ven m f actio In an attempt to dissociate the histamine releasing and phosphatidase A ctivities we tried to block one of them by heat denaturation. Aqueous sol tions of the material to be treated were enclosed in sealed glass mpoules and heated in an oven for 30 min at dif ferent temperatures. The histamine releasing and phosphatidase A activ ties of the samples were then determined. In order to get an estimate of the temperature required to prod ce the desired effect, the first series of experiments was performed with unfractionated bee venom. It was found that heating at 135 C resulted in a complete loss of phosphatidase A acti ity while the histamine releasing capac ty wa only partly affected (Fig 4 upper diagram) When F I was heated at the sam

W are greatly indebted to Professor Dr E Habermann, Würzburg, for supplying sam of bee venom phosphatidase A

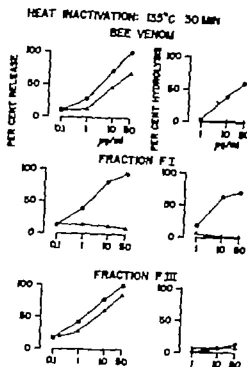


Fig. 4 Histamine releasing and phosphatidase A activities in bee venom, F I and F III after heating for 30 min at 135 C. Hydrolysis and granule incubation were run for 10 min at 37° C. ▲——▲ heated material; ●——● non-heated material.

temperature both histamine releasing and phosphatidase A activities were completely lost (Fig 4 middle diagram) In F III the same treatment had only a slight effect on the histamine releasing capacity (Fig 4 lower diagram) Thus the histamine releasing and phosphatide splitting activities of F I were not separated by this means. In addition the releasing activity of F III was found to be more heat stable than that of F I

Influence of temperature pH and EDTA Histamine release and lecithin hydrolysis induced by F I were compared at 0° 22° 37 and 47 C. A similar temperature-dependency was found for the two processes (Fig. 5)

The influence of pH on the release and hydrolysis was determined for the pH-range 5—8 The pH was adjusted by the addition of appropriate mixtures of Na_2HPO_4 and KH_2PO_4 to a final concentration of 6.5×10^{-2} M or tris and HCl to 5×10^{-2} M. No pH-optimum was found for either of the activities, both being enhanced in alkaline media (Fig. 6)

The phosphatidase A is known to be activated by Ca^{++} (Zeller 1951) In the present work both the lecithin-splitting and histamine-releasing effects were strongly inhibited by ethylenediaminetetra acetic acid (EDTA) which binds divalent cations (Table I)

It was later found that histamine release induced by F III — which lacked phosphatidase A activity — was dependent on variations in temperature and pH in about the same way as that induced by F I It was also found that the effect of F III is

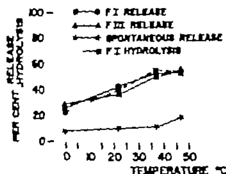


Fig 5

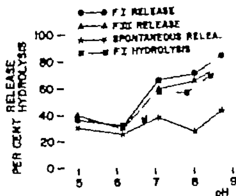


Fig 6

Fig 5. Influence of temperature on histamine release and hydrolysis. The results from one release expt. (F I 5 µg/ml F III, 5 µg/ml) and one hydrolysis expt (F I 10 µg/ml) are shown. Hydrolysis and granule incubation were run for 10 min.

Fig 6 Influence of pH on histamine release and hydrolysis. The results from one release expt (F I, 5 µg/ml F III 5 µg/ml) and one hydrolysis expt. (F I 20 µg/ml) are shown. Hydrolysis and granule incubation were run for 10 min 37° C.

Table I. Effect of EDTA on histamine release and hydrolysis. For release 5 µg/ml of F I and F III were used for hydrolysis 25 µg/ml of F I

	EDTA	Histamine release in per cent of control (2 expts.)	Hydrolysis in per cent of control (3 expts)
F I	10^{-4} M	94	120
	10^{-4} M	0	4
	10^{-4} M	0	1
F III	10^{-4} M	127	—
	10^{-4} M	37	—
	10^{-4} M	10	—

inhibited by EDTA, although to a lesser extent than that of F I (Fig. 5—6 and Table I)

Histamine release ind c d by surfac -active agents distilled water and NaCl

F action F III is directly hemolytic and liberates 5-hydroxytryptamine from rabbit platelets (Fredholm and Westerholm, to be published) as does melittin, a bee venom fraction described by Habermann (for ref see Habermann 1965) Melittin was tested in our system and found to ha releasing capacity of about the same order as F III As mel ti n is highl rface active (Habermann 1958) t might be suspected that the histamine rel asing effect of fraction F III was due to rface activity

W are greatly indebted to Professor Dr E Habermann, Wurzburg for supplying sample of melittin.

TABLE II. Surface tension (water-air interface) at 20 C. The compounds were dissolved in 0.34 M sucrose

	Concentration µg/ml	Surface tension dyn/cm
0 (0.34 M sucrose)	—	73.8
F I	1	72.9
	10	72.7
	50	58.6
F II	1	72.9
	10	71.6
	50	69.2
F III	1	72.2
	10	71.1
	50	62.5
Tween 20	1	67.4
	10	58.5
	100	43.1

The surface activity of the releasing agents was determined by measuring the surface tension in the water air interface of solutions containing different concentrations of these substances. Both the releasing fractions were found to decrease surface tension but in concentrations higher than those inducing histamine release (Table II).

The non-ionic detergents Tween 20 and Triton X 100 were found to release granular histamine although no more than about 60 per cent. The remaining 40 per cent could be released by 0.1 M NaCl, which by itself had no effect on the granules (Fig. 7).

For comparison the effect of detergents and NaCl on granules suspended in distilled water instead of sucrose was also tested. It was found that the granules spontaneously liberated about 60 per cent of their histamine upon transfer to distilled water. Tween 20 caused no further release while addition of NaCl in a final concentration of 0.1 M resulted in total histamine release (Fig. 7).

Histamine and heparin release

Fig. 8 shows that heparin — measured as radioactive sulphur — was not released from its bound state when histamine was released by bee venom. When the granules were heated in a boiling water bath both histamine and heparin appeared in the supernatant after centrifugation of the granules.

Discussion

Part of the evidence for an ionic binding of histamine in the mast cell granules is the observation that histamine is easily released from them by cations. Thon and Uvnäs (1966) who studied granules isolated after hypotonic lysis of mast cells, reported

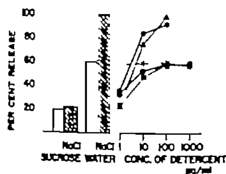


Fig 7

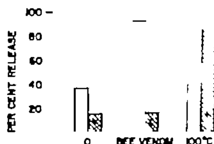


Fig 8

Fig 7 Histamine release, induced by detergents, distilled water and NaCl. Granules were incubated for 10 min at 37° C.
Left part □, "spontaneous" histamine release in sucrose and water ▨ effect of adding NaCl, 0.1 M.
Right part Concentration-response curves for histamine release induced by detergents.
Granules suspended in sucrose
●———● Tween 20 (3—4 expts.)
■ ■ Triton X-100 (1 expt.)
○———○ Tween 20+NaCl, 0.1 M (2 expts.)
▲ ▲ Triton X-100+NaCl, 0.1 M (1 expt.)
Granules suspended in distilled water
* — — * Tween 20 (1 expt.)

Fig. 8. Release of histamine □, and heparin, ▨ induced by venom, 5 µg/ml. Granules were incubated for 10 min at 37° C. As control granular destruction was achieved by heating at 100 C.

that the granules became less sensitive to cations when they were incubated in non-ionic sucrose instead of distilled water The granules used in this work, prepared by sonic disintegration of mast cells, behaved similarly when incubated in the routinely used sucrose solution they did not release histamine even when exposed to 0.3 M NaCl (unpublished observations) while they liberated all their histamine when incubated with 0.1 M NaCl in distilled water

When the granules were transferred to distilled water some 60 per cent of the histamine was released spontaneously The non-ionic detergents Tween 20 and Triton X-100 also caused a release of about 60 per cent This effect of distilled water — known to cause swelling of the granules (Thon and Uvnäs 1966) — and of surface active agents, might be explained by facilitated penetration of water into the granules, leading to a shift of the equilibrium between free and granular-bound histamine. This theory would also explain why hypotonous and decreased surface tension did not cause a total histamine release Similarly the finding that histamine remaining in the granules after such treatment was liberated on subsequent addition of NaCl, might be due to facilitated penetration of cations into the granules leading to increased cation exchange

The two histamine releasing fractions F I and F III were found to lower surface tension, and this property might thus be responsible for part of their releasing capacity It is true that the surface tension at the air-liquid interface was not significantly decreased by concentrations of F I and F III capable of releasing histamine but it

should be pointed out that the effects at this interface are not directly comparable to those at the granule-liquid interface. The surface activity of the fractions was of the same order as that reported for melittin, whose biological effects have been ascribed to its surface-active properties (Habermann 1958, 1965). This agent was found by us to have a histamine releasing effect on granules comparable to the fractions F I and F III.

Electrophoretic studies had shown F I and F III to be positively charged and thus release by ion-exchange has to be considered. Increased penetration, due to surface activity combined with ion exchange would be a possible mechanism just as detergents sensitize the granules to the action of NaCl. However both F I and F III are large molecules (according to the elution pattern on Sephadex) and the molar ratios between them and histamine too small to permit a stoichiometric exchange between histamine and positively charged groups on the F I or F III molecule.

When the phosphatidase A activity of F I was abolished by heating, or inhibited with EDTA, the histamine releasing capacity decreased. Changes in pH and temperature affected both lecithin hydrolysis and histamine release similarly. These observations suggest that the histamine releasing effect of fraction F I is due to the enzymic activity of this fraction. However according to other authors granules contain only insignificant amounts of phosphatides (Lagunoff *et al.* 1964) and therefore an enzymatic breakdown of the granular structure by phosphatidase A seems improbable. In fact the effect of F III — which lacked phosphatidase activity — was influenced in much the same way as that of F I by varying the experimental conditions. This could indicate that F I and F III induce histamine release in similar ways. Whatever effect the bee venom fractions exert on the granules, they do not seem to cause a breakdown of the granular structure leading to dissolution of the granules since heparin, which is a constituent of the granular stroma, was not released along with the histamine.

It is concluded from the present study that the histamine release from mast cell granules by bee venom is not due to the mast cell active fraction F II but to two other fractions, both surface active, one of which shows phosphatidase A activity. Whether or not either of these activities is involved in the bee venom-induced histamine release from granules remains to be established.

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Effect of Acid in Proximal and Distal Duodenal Pouches on Gastric Secretory Responses to Gastrin and Histamine[1]

By

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Abstract

Andersson, S., G. Nilsson and B. Uvnäs. *Effect of acid in proximal and distal duodenal pouches on gastric secretory responses to gastrin and histamine.* Acta physiol. scand. 1967. 71. 368—378.

In Pavlov and Heidenhain pouch dogs intestinal pouches of the duodenal bulb and distal duodenum were isolated. Perfusion of the bulbar pouch with decinormal hydrochloric acid produced a pronounced inhibition of the secretory response to gastrin in both the Pavlov and the Heidenhain pouch group. Perfusion of the distal duodenal segment with acid did not significantly inhibit the secretory response to gastrin. Instead an increase of the acid output was often seen following acid perfusion of the distal segment. Secretory responses to histamine were not significantly inhibited following bulbar acidification. The results indicate that the pH-sensitive inhibitory mechanism of the duodenum is mainly located in the duodenal bulb. The mechanism seems to be particularly effective in suppressing the gastric acid response to gastrin.

It was shown by Andersson and Uvnäs (1961) that introduction of acid solutions into an isolated pouch of the duodenal bulb strongly inhibited the postprandial secretion from Pavlov pouches. This observation suggested that the pH-sensitive inhibitory mechanism of the duodenum may be located mainly in the first part of the duodenum.

The present series of experiments was designed (1) to determine the intraduodenal extension of the pH sensitive inhibitory mechanism and (2) to study the effectiveness of bulbar acidification on secretory responses to gastrin and histamine.

Methods

Surgical procedure

17 mongrel dogs weighing 11—17 kg were prepared as follows: 9 dogs were provided with mucosal septal pouches (Pavlov type) according to a slightly modified technique described by Thomas (1942). 8 dogs were provided with Heidenhain pouches. A double mucosal septum

[1] A preliminary report of this investigation appeared in Acta physiol. Scand. 1965. 63. 191—192.

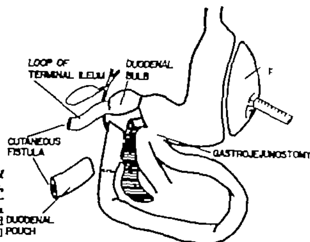


Fig. 1 Surgical preparation of the dogs: Heidenhain pouch, mucosal wall at the pylorus, gastrojejunostomy, cutaneous fistula to the isolated duodenal bulb and cutaneous fistula to the distal duodenal pouch.

was constructed at the pylorus 3—4 weeks later and the gastrointestinal passage was restored by gastrojejunostomy with the stoma in the antrum.

At third operation various procedures were performed. In all 9 Pavlov pouch dogs and in 6 Heidenhain pouch dogs the duodenum was transected just proximal to the entrance of the common bile duct. The distal end was closed and the bulb was anastomosed end to end with resected loop of the terminal ileum, which was brought to the abdominal wall to form a mucocutaneous fistula. In another 2 Heidenhain pouch dogs a duodenal pouch was constructed from 6—8 cm long segment taken just distal to the entrance of the main pancreatic duct. The proximal end of the isolated duodenal segment was closed and its distal opening was exteriorized. Such a duodenal pouch was also constructed in Pavlov and 2 Heidenhain pouch dogs, which previously had been provided with pouches of the duodenal bulb (Fig. 1).

Operations and postoperative management followed the principles of this laboratory (Uvnäs et al. 1956). Before the experiments were started the dogs were allowed a period of 3—4 weeks recovery.

Experimental procedures

Each experiment started in the forenoon after the animals had fasted 18—24 hrs. Basal gastric output was recorded for one hour and secretory responses were collected in 15 min samples. The volume was measured and the acidity determined with 0.01 N NaOH with phenolphtalein as indicator. Gastrin was prepared from hog antrum mucosa by the method of Gregory and Tracy and Gillespie (1963). Each dog received gastrin from the same batch during the entire experimental series. Doses of gastrin are expressed as grams of wet weight mucosa extracted. Doses of histamine are expressed as mg of histamine dihydrochloride.

Before the experimental series was started the dose—response curves for gastrin and histamine were determined in each dog. *Gastrin and histamine were given in doses producing* secretory rates corresponding to 1/4—1/3 of the maximal secretory responses to these stimuli. The stimulants were given in 0.9 % saline as a continuous intravenous infusion at a rate of 30 ml per hour by a calibrated peristaltic pump (Harvard Apparatus Co. Dover Mass.). In all experiments the secretory responses were followed for a control period of —3 hrs. During this time a plateau of secretion was usually reached. The intestinal pouches were then perfused for 1 hr with 0.1 N HCl, 0.9 % saline or neutral buffer solutions. After the perfusion period, the secretory output was followed for another 1 1/2 hrs. The perfusions were performed with Foley catheters, which were introduced into the intestinal pouches before the experiments started. The solutions were always warmed to body temperature before they were delivered into the intestinal pouches. The rate of perfusion was about 60 ml per hour. To insure contact of perfusion fluid with the mucosa in the deepest parts of the pouches the instilled solutions had to leave the pouches against a pressure of 5—10 cm of water. The pH of the effluent perfusate was determined each 15 min.

Statistical evaluation of data

Common methods of analysis of variance were used in the statistical calculation of results (Snedecor 1956)

Results

Effect of acid in the duodenal bulb on gastrin stimulated secretion

The effect of acidification of the duodenal bulb was studied in 27 expts. on 9 Pavlov and in 18 expts. on 6 Heidenhain pouch dogs. Reduction of the intrabulbar pH to 1 1—1 4 was consistently followed by a considerable decrease of the secretory response to gastrin (Table I and II) Inhibition generally occurred during the first 15 min period of bulbar acidification and became maximal during the two last 15 min periods of perfusion. The reduction of the secretory response was as pronounced in the Pavlov as in the Heidenhain pouch group (Table I and II) After conclusion of the perfusion the secretory response gradually returned to the control level. The return was faster in the Pavlov pouch dogs. In some experiments the acid secretion

TABLE I. Secretory responses to gastrin in Pavlov pouch dogs with and without perfusion of the duodenal bulb.

Dog no.	Number of expts.	Dose of gastrin (g wet weight mucosa)	Control hour: Range of secretory levels in meq/15 min	Control hour: Relative S.E. of mean of four 15-min periods (per cent)	Mean secretory rate in per cent of secretory level during the control hour. Half hour periods following the control hour: 1	2	3	4	5
1	3	10	0 19—0.67	8.7	18.0	7.5	21.5	69.5	95.5
2	3	10	0.17—0.20	9.0	42.0	19.5	16 0	71.5	102.5
3	3	10	0 13—0 18	9 7	57.0	27.0	37.5	103.5	113 5
4	3	10	0.20—0.28	5 4	56.5	24.0	22.5	99.0	150 0
5	3	10	0 11—0.18	12.4	12.5	1.5	4.5	8.5	77.0
6	3	10	0 44—0.51	8.8	58.0	10.0	58.5	96.0	114.5
7	3	8	0 29—0 47	10 7	64.5	14.5	23.0	101.0	130.5
8	3	8	0.20—0 38	6.1	51.5	32.5	63.0	54.0	56 0
9	3	4	0.38—1.03	7 1	66.5	34.5	49.5	65.5	76.5
Total number of experiments	27		Mean ± S.E. of mean		45.4 ± 6.5	18.6 ± 4.0	30.7 ± 18.1	74.2 ± 10.2	100 0 ± 10 0

Bold face figures represent instillation of HCl in the intestinal pouch.

TABLE II Secretory responses to gastrin in Heidenhain pouch dogs with and w..b the doudenal bulb

Dog no.	Number of expts.	Dose of gastrin (g wet weight mucosa)	Control hour: Range of secretory levels in meq/15 min	Control hour: Relative S.E. of mean of four 15 min periods (per cent)	Mean secretory rate in per cent lev l during the control hour. Half hour periods following h: 1	2	3	4	
10	3	0	0.15—0.22	7.2	46.0	33.5	40.0		
11	3	6	0.17—0.24	5.7	63.5	33.5	5		1
12	3	10	0.15—0.17	10.0	65.0	31.5	29	49.0	
13	3	6	0.19—0.55	13.7	44.0	31.5	37.5	4	4
14	3	10	0.13—0.18	16.0	31.0	1.5	3.5	10.5	
15	3	10	0.16—0.25	3.6	27.5	0	18.0	32	9.5
Total number of experiments	18		Mean ± S. E. of mean		43.5 ± 6.6	18.6 ± 6.4	26.7 ± 9.0	48.7 9	83.3 8.6

Bold face figures represent instillation of HCl in the in ex nal pouch

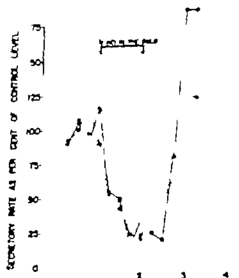


Fig. 2. The effect of bulbar acidification on secretory responses to gastrin in Pavlov pouch dog (no. 4). Each curve represents one experiment.

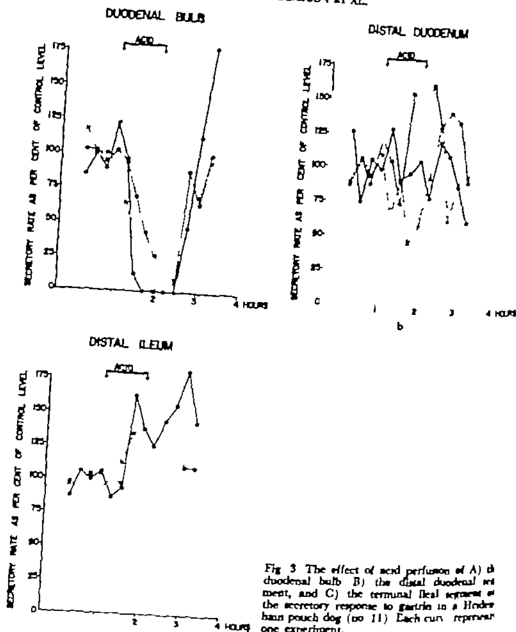


Fig 3 The effect of acid perfusion of A) th duodenal bulb B) the distal duodenal seg ment, and C) the terminal ileal segment o the secretory response to gastrin in a Heiden hain pouch dog (no 11) Each curv represent one experiment.

during the postperfusion periods increased above the control level. Representativ illustrations of the secretory pattern following acidification of the duodenal bulb in a Pavlov and in a Heidenhain pouch dog are given in Fig 2 and 3A. Control ex periments in which the duodenal bulb was perfused with saline or neutral buffer solutions have been performed. Perfusion of the bulb with such solutions did not produce any secretory inhibition.

TABLE III. Secretory responses to gastrin in Pavlov pouch dogs with and without perfusion of the distal duodenal segment

Dog no.	Number of expts.	Dose of gastrin (g wet weight mucosa)	Control hour: Range of secretory levels in meq/15 min	Control hour: Relativ. S. E. of mean of four 15-min periods (per cent)	Mean secretory rate in per cent of secretory level during the control hour. Half hour periods following the control hour: 1	2	3	4	5
8	3	8	0.16—0.31	7 4	84.0	66.5	74.5	70 5	70.5
9	5	4	0.17—0 65	10.3	121.5	124.5	138.5	161.0	147.5
Total number of experiments	8			Mean	103.8	95.5	106.5	115.8	109.0

Bold face figures represent instillation of HCl in the intestinal pouch.

TABLE IV. Secretory responses to gastrin in Heidenhain pouch dogs with and without perfusion of the distal duodenal segment

Dog no.	Number of expts.	Dose of gastrin (g wet weight mucosa)	Control hour: Range of secretory levels in meq/15 min	Control hour: Relativ. S. E. of mean of four 15-min periods (per cent	Mean secretory rate in per cent of secretory level during the control hour. Half hour periods following the control hour: 1	2	3	4	5
11	3	6	0 43—0 56	7 9	91.0	100.5	117.5	103.5	74.5
13	4	10	0 11 —0 20	12 3	87.0	107.0	103.5	57.5	83 0
16	3	10	0 14—0 20	4 0	114.5	113.5	102 0	112.0	109 0
17	5	6	0 15—0 59	8.9	101.0	100.8	141.0	134 5	1 4.0
Total number of experiments	15			Mean	98.4	105.8	116 5	106 9	98 1

Bold face figures represent instillation of HCl in the intestinal pouch.

Effect of acid in the distal duodenal segment on gastrin stimulated secretion

Acidification of the distal duodenal pouches was performed in 8 experiments on 2 Pavlov and in 14 expts. on 4 Heidenhain pouch dogs. Acid perfusion of the distal duodenal pouches caused no significant inhibition of the secretory response to gastrin in either the Pavlov or the Heidenhain pouch group. Instead, an increase of acid output was frequently observed during the second half hour period of acidification (Table III and IV, Fig. 3 B).

Effect of acid in the segment of distal ileum on gastrin stimulated secretion

With the present perfusion technique some acidification of the ileal mucosa must have occurred in the bulbar perfusion experiments. To study the influence of acidification of the ileal mucosa alone 3 expts. were performed in a Heidenhain pouch

Table V. Secretory responses to histamine in Pavlov pouch dogs with and without perfusion of the duodenal bulb

Dog no	Type of exp	Number of expts	Dose of histamine mg/hr	Control hour: Range of secretory levels in meq/15 min	Control hour: Relative S. E. of mean of four 15-min periods (per cent)	Mean secretory rate in per cent of secretory level during the control hour. Half hour periods following the control hour: 1	2	3	4
1	Histamine control	3	0 25	0 65—0 96	9.9	63.5	59 0	61 5	57.0
	Histamine and acid in the bulb	3	0 25	0 60—1.25	6.3	73.5	56.0	63 0	69.5
				Mean difference		+10 0	—3 0	+1.5	+12.5
5	Histamine control	4	0 50	0 35—0.91	4.5	91.0	93.5	88.5	89 5
	Histamine and acid in the bulb	4	0 50	0 47—0.65	5.5	87.0	76.0	78.5	81 0
				Mean difference		—7 0	—17.5	—10.0	— 8
6	Histamine control	4	0 2	0.32—0.69	7 1	105.5	83 0	90 0	101.0
	Histamine and acid in the bulb	4	0 2	0 33—0.50	11 4	112.0	107 5	113 0	121 0
				Mean difference		+6.5	+24.5	+23 0	+20 0

Bold face figures represent instillation of HCl in the intestinal pouch

TABLE VI *Secretory responses to histamine in Heidenh pouch dogs with nd of the duodenal bulb*

Dog no	Type of exp.	Number of histamine expts.	Dose of histamine mg/hr	Control hour: Range of secretory levels in meq/15 min	Control hour: R lat S E of mean of four 15-min periods (per cent)	Mean secretory rat in secretory level during the hour. Half hour periods following control hour: 1	2	3	4
14	Histamine control	3	0.25	0.22—0.3	10.5	94.0	92.5	87.0	81.5
	Histamine and acid in the bulb	3	0.25	0.14—0.28	7	**91.5**	**86.0**	84.5	96.0
			Mean difference			— 5	—6.5	—2.5	+11.5
15	Histamine control	3	0.25	0.24—0.31	4.7	89.0	97.5	89.0	106.5
	Histamine and acid in the bulb	3	0.25	0.15—0.46	4.9	**108.0**	**147.0**	105.5	110.0
			Mean difference			19.0	+49.5	+16.5	+3.5

Bold face figures represent instillation of HCl in the intestinal pouch.

dog with an isolated loop of the terminal ileum. No inhibition was observed w th acid perfusion of the ileal loop When, however the duodenal bulb t a subsequent operation was connected to the ileal loop a pronounced inhibition followed acidification of the ileal-bulbar pouch (Fig 3 A and 3 C)

Eff ct of acid in th duodenal bulb on hist m ne st m lat d se ct n

The effect of acidification of the duodenal bulb on the secretory response to histamine was studied in 11 expts. on 3 Pa lov and in 6 expts. on 2 Heidenha pouch dogs. Whereas the acid response t gastrin was quite constant, the response t histamine frequently declined after a period of time For proper evaluation of the inhibitory effect of duodenal bulb acidification on histamine-stimulated secretion control experiments had to be performed with continuous infusion of histamine during the same period of time as the acid perfusion experiments. By this means, the indications of inhibition observed in the majority of experiments w th histamine were found to be fallacious (Table V and VI)

Discussion

The effectiveness of bulbar acidification in suppressing the secretory response to exogenous gastrin compares favorably with the previous observation of Andersson and Uvnäs (1961). They found that perfusion of bulbar pouches with acid produced inhibition of the secretory response to a test meal. From their experiments it was suggested that the duodenal inhibitory mechanism may be located mainly in the duodenal bulb. The present finding, that no inhibition was achieved on acidification of the considerably larger segment of the distal duodenum strongly support this concept. Similarly Konturek and Grossman (1965) showed that acid perfusion of a proximal duodenal loop produced pronounced inhibition of responses to exogenous gastrin from Heidenhain pouches. On the other hand, they could also demonstrate some inhibition on acidification of a loop of distal duodenum. The difference between our findings and theirs may well be explained by slightly different surgical preparations of the animals. However both studies have clearly demonstrated that inhibitory influences arising from the proximal duodenum are far stronger than those inhibitory influences which could be evoked from the distal duodenum.

In accordance with the study of Konturek and Grossman (1965) no inhibition was observed following acidification of the intestinal pouch of the terminal ileum.

Secretory responses to histamine were not affected by acidification of the duodenal bulb. This confirms previous reports on the effect of duodenal inhibition on histamine-mediated responses from this laboratory (Andersson 1960). Conversely others have shown that histamine-stimulated secretion is susceptible to inhibitory influences from the duodenum (Code and Watkinson 1955, Wormsley and Grossman 1964). To date it is not possible to explain these conflicting results. However there exists experimental evidence that several factors may be of importance in interpreting the results from inhibitory studies in which histamine is used as the secretory stimulant. For example it has been shown that the degree of inhibition depends on the intensity of stimulation with histamine, inhibition decreasing with increasing doses of histamine (Code and Watkinson 1955, Wormsley and Grossman 1964). Furthermore it is known that gastrin potentiates the stimulatory effect of histamine (Passaro et al. 1963). Therefore possibilities exist that spontaneous release of endogenous gastrin may augment the secretory effect of injected histamine. Thus Andersson and Grossman (1966) showed that acidification of an isolated and vagally innervated antral pouch significantly suppressed the response to histamine from Heidenhain pouches. Therefore any inhibition of histamine-stimulated secretion observed in animals with intact antrum may be the result of interferences with the action of endogenous gastrin. For adequate evaluation of the effectiveness of the duodenal inhibitory mechanism affecting histamine-stimulated gastric secretion, the endogenous release of gastrin must be controlled and reduced to a minimum, preferably by removing the antrum. Supporting this view is the observation of Sircus (1958) that duodenal acidification did not inhibit responses to histamine in antrectomized dogs. It has also been emphasized by Shapira et al. (1960) that spontaneous depres-

ions in the secretory rate during continuous st   lat on with histan misinterpreted as evidence of inhibition if   o   u trol studies are perf present results obtained from the studies on his mi   stimulated secretic the importance of this observation. In our opin   l istamine can not be as a suitable stimulant for gastric acid secretion   st dies dealing with tl logical mechanisms of inhibition.

The bulbar inhibitory mechanism acted   eff   ively in the Heidenhain as the Pavlov pouch group of dogs, indicating that   agal   nnervation is not necessary for the inhibitory effects produced by bulbar   cidification. This finding may be taken as presumptiv   evidence for   humoral mechanism of   nhibition.

Although it has been demonstrated that duodenal inst llation of hydrochloric acid produces significant inhibition of secretory responses in gastric pouches which ha undergone both vagal and sympathetic denervation, there is still the possibility that the inhibition may be of nonspecific origin. For example distension of the duodenal bulb with acid during perfusion may cause reflex liberation of adrenal or pituitary hormons which produce   asoconstriction in the gastrointestinal tract. The different effects of bulbar acidification on secretory responses to histamine and to gastrin speak in favor of a specific inhibitory factor To prove this, inhibition must be demonstrated following acid perfusion of fully denervated pouches of the duodenal bulb.

The observation that bulba   acidification inhibits the secretory responses t exogenous gastrin suggests that the inhibitory mechanism exerts   ts effects   lose to or on, the HCl glands.

Recently   t has been demonstrated that there exists a steep pH-gradient between the duodenal bulb and the postbulbar duodenum (Andersson and Grossman 1965 Rhodes and Prestwich 1966) Values below pH 2 were not uncommon in the duodenal bulb These findings, together with our obser   tion that the bulbar mechanism may be activated at pH 3—3 5 (Nilsson 1966) indicate that the bulbar pH normally may fall to the critical pH necessary for eliciting inhibition of gastric secretion. When combined, these observations strongly suggest that inhibitory   n-fluences arising from the duodenal bulb may play an important role in the regulation of gastric acid secretion.

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An Instrument for Simultaneous Determination of Sodium and Potassium in Microsamples of Biological Material

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Abstract

Carlsson, B., E. Giacobini and S. Hovmark. *An instrument for simultaneous determination of sodium and potassium in microsamples of biological material.* Acta physiol. scand. 1967. *71.* 379—390.

An integrating microflamephotometer for simultaneous analysis of Na and K is described. The instrument has a sensitivity of $1 \times 10^{-}$ moles of Na and K and is particularly suitable for determination of these ions in nanoliter samples of biological fluids or individual cells.

The alteration of the ionic environment surrounding the isolated stretch receptor neurone of the crayfish exerts known effects (Edwards *et al.* 1963) upon the electrical properties of the cell membrane and the oxidative metabolism of the cell (Giacobini 1965, 1966, 1967). Similarly a marked reduction of oxidative metabolism is observed in the presence of ion transport inhibitors (Giacobini 1966, 1967). In the progress of these studies the need arose to measure the intracellular amounts of Na and K in individual stretch receptor neurons. The amounts of Na and K present in one cell are estimated to be in the picomol range.

With ordinary chemical techniques there is no convenient way of measuring picomole amounts and therefore other techniques which are less subject to such limitations must be considered for this purpose. These include activation analysis (Keynes and Lewis 1951), absorption spectrophotometry (Hanig and Aprison 1965), flamephotometry (Ramsay 1950, Ramsay *et al.* 1951, 1953, Muller 1958, Bott 1960, Öberg *et al.* 1967), X-ray fluorescence (Hamberger and Rockert 1961), cation sensitive glass microelectrodes (Lev 1964), helium glow photometry (Vurek 1965) and laser microprobe analysis (Rosan *et al.* 1963, Glick and Rosan 1966). Flamephotometry has certain advantages when compared to other methods since sodium and potassium give intense and easily excitable emission spectra, however, a fairly large volume is required (0.— ml). Very often only small quantities of the medium to be analyzed are available in biological work, though the element to be studied may

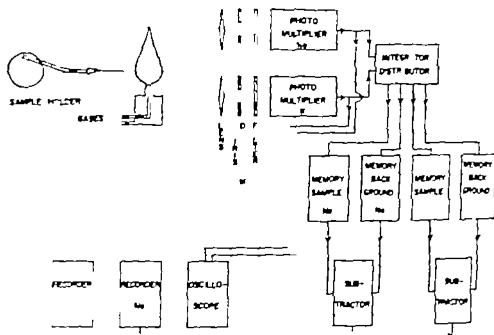


Fig. 1 Block diagram of the integrating microflamephotometer described in the text.

a major constituent of it. The dilution of the ionic content of a single cell to the required volume would therefore, exceed the detection limits of available flamephotometers. Ramsay (1950) and Müller (1958) have shown a possible solution of this problem by using the principle of integrative flamephotometry.

The instrument described in this paper represents an attempt to construct an integrating flamephotometer suitable for analysis of either individual cells or microsamples of biological material.

Description of the instrument

Principle

A schematic description of the equipment is given in Fig 1 and a general view of the equipment in Fig 2

The instrument is in principle similar to those described by previous authors. It exhibits, however some unique features.

The biological material to be examined is either sucked into a nanoliter pipette when a fluid or isolated by dissection, when a solid, and placed on a thin wire and introduced into a flame where excitation takes place. The time integral of the intensity of the light emitted at the chosen analytical spectral wavelength of an element is proportional to the amount of element present (Ramsay 1950 Müller 1958) The emitted light pulse is transmitted through selective interference filters to two

[1] A preliminary report of this work has appeared in Acta Physiol. Scand. 1966, 68 Suppl. 277 p. 32. See also Giacobini, E. 196

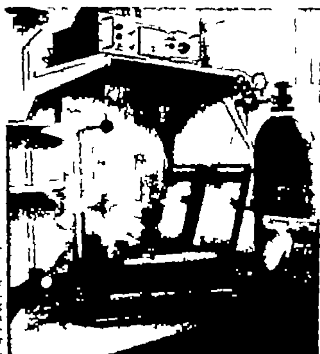


Fig. 2. General view of the integrating microflamephotometer. From left to right, sample carrier burner, the two photomultiplier tubes, double beam oscilloscope. Upper middle, gas regulating system. Above, the stabilized high voltage unit, the integrating circuit and the program unit. Upper right, the two pen servorecorders.

photomultiplier tubes as Na and K respectively. The output signals are displayed on a double beam oscilloscope and electronically integrated simultaneously. The signals show a characteristic pattern which makes detection of improper excitation possible. The integrated signals are then fed into capacitor memories and the background is subtracted utilizing a second memory which received an integrated signal arising from the sample free wire which is introduced into the flame a second time. The two signals (sample and background) are read out through a subtractor and the result is registered on a potentiometric recorder.

Gas regulation and flame

One of the most important features in the successful operation of the instrument is the composition, structure and temperature of the flame. The flame must be highly reproducible and constant so that those characteristics which can affect the emission process do not change during the analytical test. Our system for controlling and monitoring the oxidant and fuel flow to the flame was found to be quite satisfactory. A setting made in the morning needed no adjustment throughout the day.

Different flames were tested. Generally it was found that any of the different hydro-carbon flames used by the other authors had some disadvantages. First, they gave a relatively high background, second they produced deposits on the wire used for the introduction of the sample. Both of these disadvantages may greatly interfere with the reproducibility of the operations.

Among the different flames tested the air-hydrogen diffusion flame was found to be the most suitabl It was not very luminous and ga e a favourable background. By adding either oxygen or nitrogen there was the possibility of varying the temperature over a wide range. Since we were concerned with sodium and potassium, and these elements with low ionization potential, are most easily excited, we were not interested in obtaining a high flame temperature. A relatively cool flame was preferable since we were working with biological material containing many other elements beside Na and K. In a cool flame these are less prone to be excited and to cause troublesome interference. It is obvious that the smaller the flame the less light emitted and the emission of the introduced sample may be observed more readily Our flame was about 15 mm high. The bulk of burned flame gases must, however provide sufficient thermal energy to optimally vaporize and dissociate the sample in order to produce the neutral atoms which are the potential emitting species. When working in the 10^{-11} mole range the usual setting was a hydrogen pressure of about 65 mmHg A standard manufactured hydrogen was used containing only 30 p.p.m. of impurities, mainly oxygen. A reproducible and steady flame was obtained by using the following regulating system. A standard high pressure regulator reduced the tank pressure to about 1.5 kg/cm To remove the dust a Millipore gas line filter (0 45 μ pore diam) was installed before the low pressure regulator The latter was provided with a thin membrane of large diameter (Dräger Lübeck) Betwen the regulator and the burner a mercury manometer was inserted (Fig 2) The most usual setting was 65 mm/Hg. Air was supplied from the laboratory lines. A low pressure regulator for air (Dräger Lübeck) and a flow meter (AGA, Stockholm) were used to monitor the flow to the burner (Fig. 2)

Burner (Fig 3)

The burner consisted of a 7 cm high thin walled steel pipe the nozzle being 1 mm in diameter The pipe, connected to the hydrogen line, was placed in the centre of an equally high blackened steel tube, 2.5 cm diameter At the bottom of this was a holder for a Millipore filter (0 45 μ pore diam.) Through this, dust free air is pressed, forming a cylindric flow surrounding the flame. (At an air flow exceeding 1 l/min no dust from the surrounding air can reach the flame) Concentric to the nozzle was another steel cylinder (5 mm diam.) with a narrow slit for the passage of the sample. This cylinder prevented the light emitted by the glowing wire from reaching the light detecting system

Sample carrier (Fig 3)

The uniform feeding of the analytical sample into the flame for excitation is an extremely important factor for the accuracy of every photochemical determination.

The sample was placed on the tip of a 50 μ thick and 3 mm long platinum-iridium (80 20 %) wire which was mounted as an extension of a steel rod and attached to a precision clutch on th carrier This made it possible to always place the wire in the same position in rel tion to the flame and the optical system. Once

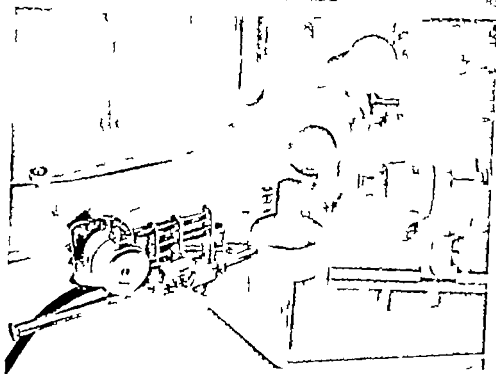


Fig 3 Sample carrier injection motor and sample holder w th the platinum ire (lef) Burner (middle) and photomultipliers (right (The house surround ng the burner has been removed)

the motordriven sample carrier was started th rest of the operation took place automatically This was achieved by means of a microsw tch directed performance control. J st before the sample reached the flame integration of the voltage outputs of the photomultipliers started. The integration time was 1 sec. The empty wire wa first withdrawn and then ad anced into the flame giving rise to a new signal which represented the background Platinum-iridium wire was choosen among other alloys for several reasons First t mainta ned ts elastic properties d ring the rough treat ment to which it was subjected d ring the numerous passages into the flame Second t was not bent during the necessary handling for the collection of samples. Third, ery th n wires could be used which reduced the distortion of the flame geometry when the sample was introduced. Fig 8 A show that when using a 100 / thick wire the excitation f the sample was not niform The second olley arose when the wire was heated to ncandescence This fact impairs th reproducibil ty of the ex periment Fig 8 B shows that by changing to a 50 μ thick wire th excitation proc ess was less subjected to such errors The fact that potassium was less affected tha sodi m may be due to difference in melting points. Another factor determining the choice of the alloy used was the unspecific light emitted by the glow ng wire This

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diaphragm which was located in front of an interference filter (Schott & Gen., Mainz) For sodium the filter had a T_m (maximal transmittance) of 60 % at 586 nm and a HW (half intensity bandwidth) of 18 nm. For the potassium channel at 764 nm, T_m is 59 % and HW is 15 nm. The transmitted light falls on the cathodes of the photomultiplier tubes, which were kept at constant and optimal working temperature by a thermostatic regulated system. Many workers have made use of monochromators. This demands skilled optical engineering. We are concerned with a relatively cool flame and filters having good specifications are easily available. Thus, it is an advantage to avoid the much more complicated use of a monochromator for obtaining adequate resolution of the desired wave length.

Electronics (Fig. 1 and 4)

The electronic part of the instrument common to both Na and K channels consists of the following units a) program unit, b) stabilized high voltage unit (Oltronix LS 24 R) c) low voltage stabilizer for the three operational amplifiers (Philbric P 65 AU) of each channel. Fig. 4 shows only one of the photomultiplier units.

The photomultiplier for the K channel is EMI 9558 B and for the Na channel RCA 7102.

The program unit, which is started by push button, takes care of the proper timing for the resetting relay (RL) the injection motor and the rotary stepping switch. To avoid any manual manipulation the operation is done entirely automatically.

In the first step the rotary stepping switch is activated and moves from resting position 1 to position 2.

When the platinum wire carrying the sample reaches the flame the relay RL is activated, the connections RL 1 and RL 2 are opened and RL 3 is closed. Now the integrator is open to register the voltage output of the photomultipliers which are simultaneously displayed on dubble beam oscilloscope. The integrator is followed by an inverting amplifier which is necessary to obtain the right polarity for the following subtractor circuit. In order to obtain the highest possible resolution a variable gain from 1 to 200 is used by changing the ratio R 5 to any of the resistors from R 6 to R 10.

The amplified and inverted signal is then stored by charging the condensor C 3 via RL 3 and the rotary switch.

After the sample has been burned the injector returns to the starting position and at the same time opens RL 3 and closes RL 1 and RL 2.

The integrator is now reset. The stepping switch moves to position 3 and the entire sequence is repeated with a blank to register the background signal. The signal is stored by charging condensor C 2.

The stepping switch moves to position 4 then the charge of C 2 is subtracted from the charge of C 3. The result is registered on a pen servorecorder via a divider (R 16 and R 17) The rotary stepping switch finally returns to resting position 1

The use of photomultipliers offers definite advantages when very small amounts

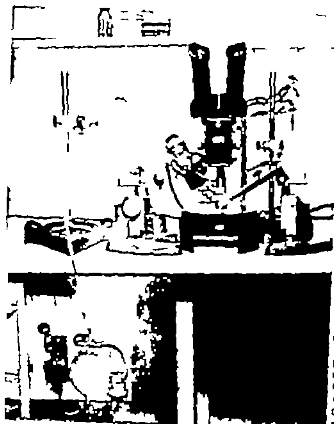


Fig. 5. Assembly for handling the sample. Notice the driving syringe (lower left) connected to a plastic t be filled ith oil to the nanoliter constriction pipette. Container with the sample (middle) and sample holder in the end of which the platinum wire is ttached (right)

an ion must be measured, but require very high sensitivity Using the equipment described here an indi idual nerv cell weighing about 0.2 µg wet weight gives an output s gnal p to 200 mV (Na) and 2 V (K) Such a high signal level makes it possible to use amplifiers and components of commercial grade.

In our experience this has gi en negligible drift.

Handl ng of th ampl (Fig 5

For measuring small v l mes f fluid or for making calibration curves we used nanoliter constriction pipettes A detailed description of how to construct them is given by De Fonbrune (1949) Briefl th end of a 10 cm long Pyrex glass t be (3 mm int diam.) was pulled out to g pillars of about 300 µ m diameter Further pulling and final forming of the pipett w made in the microforge A series of pipettes with volumes ranging between 0 d 20 nl was used. Their calibration was made in the Farrand fluorimeter us ng sol tions of quinine hydrobromide The large open end of the pipette was connected n oil-filled plastic tube to a driving syringe and mounted in a micromanip lat r F g 5) Pipetting was done under the control of a dissection microscope at about 80 and the volume was delivered directly on the tip of the platinum-iridium w re Solid samples i.e single cells were placed

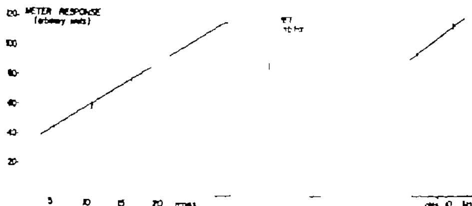


Fig. 6. Standard curve for potassium. Ea h ser he es. Vertical bars indicate ± S.E.
Fig. 7. Standard curv for sodium. Each to the s. Vertical bars indicate ± S.E.

directly on the tip of the wire afte so t F l so ion Ca taken to a oid contamination with dust since dust pa t les ontain enough of the elements t be measured to severely disturb the read ng

Other methods of estimating sampl ol me F been sed Muller (1958) and Oberg *et al.* (1967) estimated the l me u ple l g the diameter of a small sphere formed when the sampl is deposi ed il These a thors point out, however that the reproducibility of this method is affected when the samples are small. This is partly due to error in measuring me nd partl due to the effect of the oil on the sample.

Analysis of standards and biologic l material

An example of standard curves for K nd N is shown in Fig 6 and 7 when the equipment is operating close to maximum sensitivity It ca be seen that the curves are linear in the range between 2.7 10 ¹² and 7 10 moles Ea h point represents the mean of several determinations for the indicated N nd K content of th samples. The vertical bars show the standard error of the mean. The lowest detected amount of Na and K was 1 10 moles The coefficient of ariation depends pon the range in which the instrument is operating. For N it lies between 5 and 12 per cent and for K it lies between 4 and 10 per cent. In judging these figures one ha to consider the analytical procedure as an entity The coefficient of ariation for the ratio between each reading in simultaneous analysis of 2.7 10 moles of Na and K is 2.4 per cent. This indicates that the error is a result of inaccurate measurement of the delivered volume. However these figures are high compared to macroscale flamephotometrical methods in which the coefficient of ariation may be between 0.2 and 5 per cent.

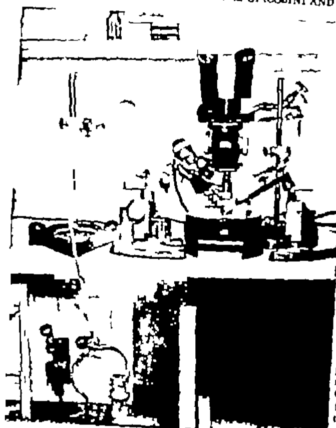


Fig 5 Assembly for handling the sample. Notice the driving syringe (lower left) connected [illegible] plastic tube filled [illegible] oil to the nanoliter constriction pipette Container with the sample (middle) and sample holder in the end of which the platinum wire is attached (right)

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Acta physiol. scand. 1967 71 391—400

From the Department of Pharmacology Karolinska Institutet Stockholm, Sweden

Intracellular Variations of Na and K in Isolated Nerve Cells

By

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Abstract

GIACOBINI, E., S. HOVMARK and Z. KOMETIANI. *Intracellular variations of Na and K in isolated nerve cells* Acta physiol. scand. 1967 71 391—400.

The variations of intracellular Na and K content in isolated crustacean sensory neurons were examined in different experimental conditions with the help of a sensitive microflamephotometric technique. In the experiments with physiological solution, after the isolation of the sensory neuron, the ionic events can be divided into two periods. During the first period, the initial period, a continuous recovery from the initial distortion of the ionic contents is going on. The intracellular cationic content is influenced during this period by at least two processes acting simultaneously and counteracting each other one depending on the manipulation of the cell, the other on the active transport. If the active transport mechanism is inhibited, (e.g. under the effect of ouabain or in K free or Na free medium) the recovery does not take place. The second period starts about 1 hr later and is characterized by stationary levels of Na and K which — in Van Harreveld's solution — are close to physiological levels. This period may continue during at least 5 hrs. Tetrodotoxin (0.0167 μg/ml) glucose (1 mM) and shortlasting physiological stimulation (about 30,000 impulses) do not significantly change the intracellular Na or K.

The analysis of mechanisms of ion transport in the neuron should be facilitated if measurements of intracellular ionic contents could be carried out

Considerable technical difficulties are however still encountered in such measurements. The available techniques do not have the required sensitivity do not allow discrimination between different ionic compartments and furthermore experimental changes of the ionic environment are not readily controlled

The ideal biological model should be constituted by a cellular unit completely isolated and maintained in physiological conditions of excitability Most of the cell preparations at our disposal do not however fulfill these requisites. They are not easily isolated and the dissection procedure causes severe injuries to the neural and axonal plasmalemma. This often gives rise to irreversible damage and distortion of ionic concentrations

Due to these difficulties most of the studies on cellular ionic movements in the nervous system have been performed in slices of cortex or in isolated axons of amphibia or invertebrates.

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The nerve cell isolated from the crustacean stretch receptor organ (CSRO) is, from this point of view probably the most suitable single neuronal preparation. The electrical properties of the membrane of this cell have been subjected to thorough studies (Terzuolo and Washizu 1962, Edwards *et al.* 1963) as well as to metabolic studies (Giacobini *et al.* 1963, Giacobini 1965, Giacobini and Grasso 1966).

Compared to isolated axons, the CSRO has the obvious advantage of representing a good model of the whole neuron in which the study of several parameters of functional activity including generation of impulse activity is feasible.

The object of this work is to characterize some of the mechanisms which are responsible for the maintenance of the intracellular concentration of cations in isolated nerve cell preparations.

The present study therefore involves changes in Na and K content

a) occurring during the initial period following isolation and exposure of the cell to the bathing medium. This aspect has not been earlier investigated in isolated cell preparations.

b) subsequent to the initial period of incubation and in conditions during which movements of Na and K are reduced. The latter effects are obtained either by varying the exterior ionic concentration of Na and K or by means of selective inhibitors.

c) after short lasting periods of excitation by physiological stimulation.

The recent development of a sensitive microflamephotometric technique (Carlson *et al.* 1966, 1967) makes it possible to determine the intracellular content of Na and K in isolated slow adapting stretch receptor neurons under the above experimental conditions.

Material and methods

Preparation of isolated cells

The slowly adapting stretch receptor organs used in this investigation were dissected from abdominal segment of crayfish (*Astacus fluviatilis*). Only young specimens were used measuring about 5—8 cm in total body length. The animals were collected during spring and autumn and kept in the dark in a large tank containing circulating fresh lake water at constant temperature (about 17–18 °C). Oxygen was constantly bubbled through the water.

The animals were fed fish meat until they were transferred from the tank to the laboratory. In the period preceding the experiment (generally 1 or 2 weeks), the crayfish were maintained in smaller tanks in the same conditions as above except that they were starved. All experiments were performed at 17–18 °C.

A single receptor cell (slowly adapting cell) was isolated and suspended in a small petri dish containing 2 ml of van Harreveld solution. The muscle bundle was mounted on a mechanical stretcher so that different degrees of stretch could be applied to the muscle. Impulse activity was recorded by means of a 30 μ thick wire electrode and displayed on an oscilloscope and audiomonitored. The total number of impulses as well as the impulse frequency was recorded on a digital counter. After a number of impulses the cell was trimmed to the standard dimensions, whilst in the mechanical stretcher, and was quickly either transferred to the new medium or to the sample carrier for the analysis by means of a hair point.

The final preparation consisted of the standard specimen having the minimal dimension still compatible with function (cell body of the neurone of the slowly adapting stretch receptor organ, a segment of axon approximately 1600 μ long and 400 μ portion of the muscle bundle where the dendrites are imbedded) as described by Giacobini *et al.* (1963). Particular care was taken to separate the cell preparation from other axons and surrounding glial cells.

Determination of Na and K

The integrating flamephotometer which was previously described by Carls[...] (1967) was used for determination of Na and K. Only its principle will be [...] here.

The material to be examined is placed on 50 μ thick wire and introdu[...] here excitation takes place. The time integral of the intensity of the ligh[...] the chosen analytical spectral wavelength of an element is proportional to be amou[...] element present. The emitted light pulse is transmitted through selectiv interference fil[...] to two photomultiplier tubes as N[...] nd K respectiv[...] Their outpu signals are electronically [...]grated. The integrated signals are then fed in capacitor memories and the background is subtracted utilizing second memory which recei ed an integrated signal arising from the sample-free wire which is introduced int the flame second time The two signals sample and background) are read out through subtractor and the result registered on potentiometric recorder In these experiments the setting of the instrumen was hydrogen pressure of 35 mm/Hg and an air flow of 2 l/min.

The calibration of the instrument was made in the following way Samples containing known amounts of Na and K were analyzed. The ratio between the amounts of Na and K were plotted against the ratio of the readings obtained

The mathematical analysis of the experimental dat as accomplished by the statistical method for determining linear regression (Goulden 1939) nd it was determined that two linear functions with diff rent coefficients of regression provided the best f t as shown in Fig 1

Solutions

The different solutions used were made p in the following w y

Physiological saline [...]*eferred t as* [...]*ormal sol tio* 205 mM N Cl 5.4 mM KCl, 13.5 mM $CaCl_2$ 2.6 mM $MgCl_2$, 2.7 mM $NaHCO_3$ (Van Harreveld 1936)

Sodium f ee medium. NaCl and $NaHCO_3$ were replaced by equimolar amou ts of choline chloride and $KHCO_3$ respectiv ly

Potassium f ee medium KCl was replaced by an equimolar amoun of choline chloride.

T examine the effect of glucose and ouabain, these compounds were added to physiological

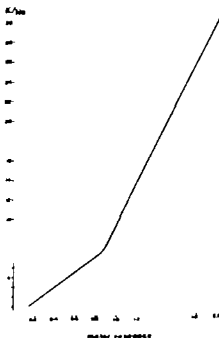


Fig 1 Relationship between K/N ratio and corresponding meter response calculated as described in he text.

solutions to give a concentration of 1 mM. The effect of tetrodotoxin (Sankyo Co., Tok.) was examined using a physiological medium containing 0.83 μg/l or 16.7 μg/l.

Washing solution 179 mM choline chloride 50 mM Tris-HCl buffer

All solutions were adjusted to pH 7.5 with 0.1 NHCl or Tris and isotonicity was maintained.

Washi g

The cell was washed in order to remove the dhering saline medium. An optimal washing procedure is essential before the analysis. All Na and K adhering to the cell must be removed and at the same time intracellular ion flow outwardly across the cell membrane must be minimized. A washing procedure was therefore established to give maximal readings for Na and K with a corresponding maximal ratio between K/Na.

After dissection and trimming, the cell was quickly transferred with a hair point from the normal to the washing solution. Here it was immersed for 43 sec and then picked up on the tip of the 50 μ thick wire and gently shaked in the solution during 45 sec. This wire carrying the cell on its tip was placed in the sample carrier of the flamephotometer. The solution was allowed to evaporate during 30 sec before the analysis was started.

Results

Although measurements of absolute amounts of Na and K per unit weight are feasible with the described technique no attempt was made here to obtain absolute values.

The parameters studied during the different experimental conditions were the ratio between Na and K and the relative changes in the total amounts of Na and K in single cells.

Fig. 2 is based on the analysis of 42 cells. It shows that the ratio K/Na has less deviation from the mean than the total amounts of Na and K.

From this figure it can be calculated that about 70 per cent of the cells will not deviate more than ± 5 per cent from the mean K/Na ratio whereas the corresponding percentage for total amounts of Na and K is 20—30. Therefore, the K/Na ratio is the more reliable parameter because the volume of different cells is different and therefore contributes to the deviation of the total amounts of Na and K from the mean.

This means that relative changes of Na and K can be estimated without determination of cellular volumes. This represents an advantage since in this type of experiment the sample has to be analyzed as quickly as possible after trimming and no suitable method rapid enough for determination of cell volume under these conditions is available.

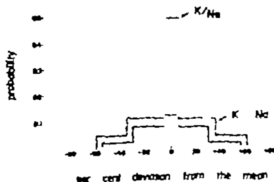


Fig. 2. Relationship between probability (relative frequence) and per cent deviation from the mean for the K/Na ratio and the relative amounts of K and Na.

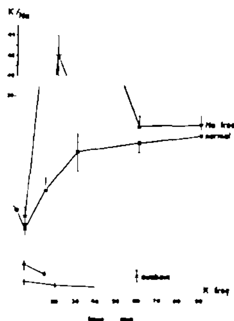


Fig 3. Variations of the K/N ratio in diff rent experimental conditions. Vertical bars indicate ± S E. of the mean.

The following results are reported Fig 3 4 and 5 In these figures each point represents the mean of 12—18 determinations in different cells. Vertical bars indicate the S E. of the mean.

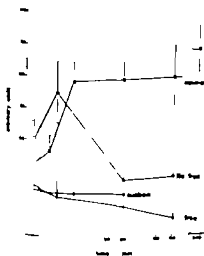


Fig. 4

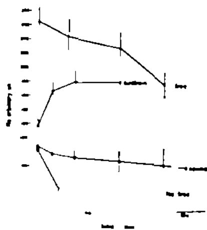


Fig 5

Fig 4 Relativ hanges of K in arbitrary nits in different experimental condition Vert al bars indicate ± S E. of the mean.

Fig 5. Relativ hanges of N in arbitrary units in different experimental conditions. Vertical bars indicate ± S E of the mean

1 Changes in Na and K content occurring after isolation and exposure of the cell to the physiological incubation medium

The K/Na ratio decreases during the first 5 min following isolation of the cell. This decrease is due to a simultaneous fall in Na and K. The former is however not so pronounced. After this first period the K/Na ratio increases steadily d ring the period from 5 min to 90 min. At 60 min of incubation in normal solution the K/Na ratio is 15 The K values increase during the period from 5 to 30 min when they reach a steady state. After that they again slowly increase to 300 min. Na decreases, first rapidly between 5 and 30 min and then very slowly to 300 min after dissection.

The events are therefore characterized by a first phase lasting about 5 min during which the intracellular contents of Na and K are reduced and by a second phase during which the K content of the cell increases whilst the Na content is maintained approximately constant.

2. Incubation in ouabain 1 mM

After a period of 5 min in ouabain the K/Na ratio is somewhat lower than in the normal solution, due to a fall in K and an increase in Na. The K/Na ratio falls slowly during the next 10 min and afterwards is maintained constant. The intracellular K falls very slowly or maintains itself almost constant while Na increases, at first rapidly during the period from 5 to 30 min and it is then constant up to 60 min. The final result of the incubation in ouabain is a decrease of the K Na ratio from 15 to 2. This is due to a reduced content of intracellular K and a pronounced increase in intracellular Na as compared to the corresponding values in normal solution.

3 Incubation in K fr e medium

The absence of K in the surrounding medium medium lowered the ratio K Na after ,5 min. due to a fall in K and a pronounced increase in Na. From 5 to 90 min after isolation the K Na ratio falls slightly The K/Na ratio is therefore reduced from 16 (normal sol tion) to 0.5 During this period the intracellular K falls steadily while the Na content also falls but more rapidly The final situation is a marked reduction in K and a marked increase in Na in the cell, compared with the corresponding values in normal sol tion.

4 Incubati n n Na fr e medium

No changes were detectable in the K Na ratio after 5 min incubation in Na-free medium. The K Na, however increases very rapidly during the period from 5 to 90 min, where it reaches its peak. From this moment to 60 min after incubation the ratio falls steadily and from 60 min to 90 min the K Na ratio of the cell is maintained constant. At 90 min the K Na ratio is not significantly different from the normal.

The behaviour of the intracellular K is characterized by two phases. During the first phase (from 5 to 20 min) the K alues increase and during the second phase

Table I. Variation of K/Na, K and Na expressed in arbitrary units during differen experimental conditions

Experimental conditions	K/Na	K	Na
Control	15.3±0.9	99±13	66±12
Tetrodotoxin 0.0167 µg/ml (60 min)	15.0±2.2	106±15	68± 6
Glucose 1 mM	15.6±1	87±13	56± 5
Physiological stimulation (35 000 impulses±5 000)	13.8±2.4	97± 5	68±10

from 20 to 60 min, they decrease. After this time the K content remains constant for a further 20 min. The Na values decrease rapidl from 5 to 20 min and after this period remain practically constant.

The final result is that both Na and K in the Na-free medium are markedly reduced if compared with the corresponding values in normal solution. However the reduction is more pronounced for K than for Na.

3. Incubatio in tetrodotoxin (0,0167 µg/ml) glucose (1 mM) and after short lasting physiological stimulation.

As seen in Table I exposure to tetrodotoxin for a period of 60 minutes had no effect. Similarly the addition of glucose (60 min) or a short lasting timulation (mechanical stretch) of about 60 min (about 30.000 impulses) did not significantly change the intracellular Na or K.

Discussion

The results presented in Fig. 3 4 5 indicate that the ionic events can be divided into two periods. The first period starts from the moment that the cell is trimmed and lasts for about 30 min. It is referred to as the initial or recovery period. This period can be subdivided into two different phases the first lasting a few minutes, in general 5 min and the second about 30 min. The second period starts immediately after the first, that is after 30—60 min, and is referred to as the tationary period.

The initial period does not reflect physiological condition but changes in the diffusion barriers, probably due to the manipulation.

It has been reported (Van der Kloot 1966) that both the heart and somatic muscle of crayfish gain substantial amounts of Na when dissected and placed in Van Harreveld's solution. The somatic muscle also lose K.

We visualize the injury made by our dissection technique as discontinuities in the neuron plasmalemma and axolemma through which ions can readily pass along their concentration gradients. This situation is clearly reflected by the values obtained in the first phase of the recovery period.

It is known from experiments on single axons that the damage to the neural plasmalemma can be repaired in a relatively short time by a partial reconstit tion of

the membrane structures of the axon (Lubinska 1956) We may therefore, assume that during the initial period the discontinuities are progressively closed Simultaneously the active transport mechanism restores the cell to the normal condition.

When the stationary period has been reached the active transport mechanism is sufficient to maintain a steady state In our opinion, this period reflects a situation close to physiological conditions in the intact cell. The ratio K/Na found in our experiment after incubation in physiological saline medium for 60 minutes agrees with the values reported in the crayfish *Procambarus Clarki* by Wallin (1967)

In judging the results obtained with varying external ion concentrations, or after the application of inhibitors, several factors must be considered. The most important are the concentration gradient of Na and K across the cell membrane, the mechanism of active transport and the injury produced by the mechanical manipulation during the dissection procedure.

The most evident variation of ionic content was after treatment with ouabain — a specific inhibitor of ion transport and ATPase activity The normal K/Na ratio fell from 15 to 2. This was due to a reduction in intracellular K and increase in intracellular Na — clearly showing that the recovery process is not capable of maintaining the physiological level of these ions. This block of active transport is well known from several earlier investigations. In the squid giant axon, (Caldwell and Keynes 1959) 10^{-4}M ouabain reduced the rate constant of Na efflux to 10—30 per cent of its normal value.

When the cell is incubated in K free solution the same final effect (during the stationary period) is obtained as with ouabain. It is known (Hodgkin and Keynes 1955) that the absence of K in the bathing medium considerably depresses the mechanism for the active extrusion of Na ions in invertebrate nerves.

Furthermore K and ouabain are competing with each other for the K-acti e site of microsomal ATPase (Skou 1965) therefore this effect can be ascribed to inhibition of the ionic pump.

If Na or K are absent from the medium, the cells lose their Na content without a corresponding uptake of K (Fig 4 and 5) In the case of a Na-free medium this could be explained by passive diffusion. However this explanation cannot be valid for the loss of Na in the K-free medium. As seen in Fig. 5 some Na moves against an electrochemical gradient so that it must be actively transported in the absence of transport of K. As earlier pointed out, in nerve cells of *Sepia* and *Loligo* (Hodgkin and Keynes 1965) and in rat liver cells (Elshove and Van Ross in 1963) the coupling between Na and K transport is close, but not absolute Probably the crayfish neuron constitutes another example of such a loose coupling

From the above it appears that the processes are more complicated during the initial period since at least two perhaps more processes are taking place simultaneously and counteracting each other It must also be borne in mind that our preparation may consist of several membranes with different characteristics and several ionic compartments. In spit of all the care taken during dissection some of the glial satellites may still be sticking to the preparation. The sheath cells (Schwann cells)

surrounding the nerve fibre of the squid have higher Na concentrations and lower K concentrations than the axon (Villegas *et al.* 1965). This suggests that the Schwann cells or the glial Na may help to maintain adequate ionic concentrations for active transport in the plasmalemma or axolemma-glia cell space during a certain period. The more than 40 per cent reduction in O consumption of the crayfish neuron when subjected to ouabain (10^{-4}M) or in Na free or K free medium (Giacobini 1965, 1966) supports the involvement of an energy dependent active transport mechanism.

Tetrodotoxin, a selective inhibitor of both inwardly and outwardly directed passive Na movements (for ref see Kao 1966) had no effect on the Na and K levels in our cell. This result is of interest since to our knowledge, no measurements of intracellular cations have been reported in isolated cells or axons during exposure to this compound. McIlwain (1967) reported that tetrodotoxin has no effect upon unstimulated mammalian cerebral tissues.

The brief physiological stimulation applied to our preparation did not have any effect on the ionic content.

Conclusion

The results of this investigation demonstrate that the analytical method and the cell preparation used are adequate for studying the variations of Na and K levels in single and isolated neurons under different experimental conditions. After the isolation of a crustacean stretch receptor neuron the ionic events can be divided into two periods. The first period is characterized by a continuous recovery from the initial distortion of the ionic content. The recovery does not take place if the active transport mechanism is inhibited. The second period starts about one hour later and is characterized by stationary levels of Na and K, which — in normal solution — are close to physiological levels. This period may continue for at least 5 hrs.

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Adrenergic Reinnervation of Anterior Chamber Transplants

By

TORBJÖRN MALMFORS and LARS OLSON

Reappearance of noradrenaline (NA) in the denervated sheep heart as result of ingrowth of regenerating adrenergic nerves was first shown by Goodall (1951)

Growth of the sympathetic adrenergic neurons has also been studied with the Falck—Hillarp fluorescence technique for histochemical visualization of monoamines under various experimental conditions. Thus, ingrowth of noradrenaline nerve terminals into pineal gland transplants was observed by Owman (1964)

Little attention, however has been given t the organization of newly formed sympathetic innervation apparatuses of transplanted tissues and organs. An investigation is therefore now being carried out to elucidate the development and appearance of such nerves in different grafted tissues.

Small pieces of various organs were aseptically transplanted to the anterior chamber of the eye of adult Sprague-Dawley rats. Autologous, homologous and heterologous grafts (from A.M.R.L.-mice) were used. Host eyes and grafts were observed daily ra it with dissecting microscope and the animals sacrificed after different time intervals. Transplants and irides of the host eyes were then prepared for fluorescence microscopy as described earlier (Norberg and Hamberger 1964 Malmfors 1965 see also Corrodi and Jonsson 1967)

Autologous iris graft The first few days postoperatively no fluorescent nerves could be detected in the graft as a consequence of its surgical denervation during the operation procedure. From the fourth day on, when vascular contacts with the host iris could first be observed, NA-containing nerve fibers were also detected running from the sphincter border and anterior surface of the host iris in tissue strands over to the graft (Fig. 1) Single nerves began to arborize over the dilator area of the graft and became somewhat more numerous after one week (Fig 2) These ingrowing nerve fibers were relatively thin and weakly fluorescent with a bulgy appearance and their growing tips were often enlarged and stronger fluorescent.

During the second and third week a continuous plexus of adrenergic nerve terminals was established in the graft (Fig. 3) It resembled the normal adrenergic ground plexus of irides as described earlier (Malmfors 1965) Thus, it often contained several nerve terminals running together in each strand of the plexus. The meshes of the network were larger however and the structural organization was less regular Large bundles of bulgy non-terminal axons were frequently seen in the graft. Such highly fluorescent non-terminal axons could simultaneously be observed in the connective tissue bridges between the host iris and the graft and in the host iris itself A slight increase in the density of the ground plexus occurred during the fourth week. Thus, the intact sympathetic adrenergic neurons can easily extend their innervation areas.

Pharmacological studies according to Malmfors (1965) demonstrated a normally functioning membrane mechanism for NA uptake of the newly formed adrenergic nerves as well as a normal release of the transmittor upon nerve stimulation. Studies of NA uptake after pretreatment with H 44/68 the methylester of α-methyl-paratyrosine an inhibitor of NA synthesis, indicate that the bulgy appearance of the non-terminal axons is due to aggregations of amine storage granules

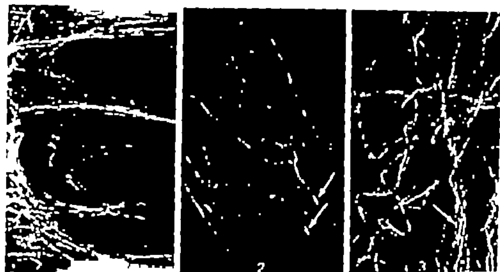


Fig. 1—3. Stretch preparations of rat irides. Fluorescence microphotographs, ×190. 1. Bundles of nerve fibers bridging over from host iris (sphincter border at far left) towards the graft. Note bulgy appearance of nerves. 2. Graft, one week. Singular nerves arborizing over the dilator area. Enlarged growing tips (↑). 3. Graft three weeks. A well developed adrenergic ground plexus. Large bundle of non-terminal axons (↑).

Other types of grafts. Homologous iris grafts showed about the same extent of re-innervation after 14 days as did the autologous grafts. Even iris grafts from mice received an adrenergic innervation from the rat host irides but to a much less extent. Interestingly adrenergic iris nerves were also able to innervate slices of the submaxillary gland as well as the auricles of heart and the vas deferens. This implies that the sympathetic nerves from the superior cervical ganglion can also innervate effector organs normally innervated by other sympathetic ganglia.

Furthermore, if the superior cervical ganglion was ipsilaterally transplanted to the anterior chamber it vigorously reinnervated the now denervated recipient iris. Obviously, therefore, normal nerve impulse activity is not necessary for this re-innervation and the outgrowth can take place in spite of the axotomy performed close to the nerve cell bodies.

The results thus demonstrate a pronounced ability of sympathetic neurons to reinnervate denervated tissues.

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Monoamine-Storing Cells of the Enterochromaffin Type in Gastrointestinal Tract of Human Fe

By

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5-Hydroxytryptamine (5-HT) in the gastrointestinal tract is present in a large of enterochromaffin cells (Erspamer 1966). Another system of morphologically si lar cells has been found to be the major storage site of gastric histamine in the r (Håkanson and Owman 1967 Th nberg 1967). These enterochromaffin like lls do not contain 5-HT but are capabl of synthesizing and storing this and ther monoamines upon administration of their respect ve precursors (Håkanson et al 1967a). In the rat, both cell systems ppear even before birth (Håkanson t al 1967b). In the present report demonstrates that these two types of enterochromaff cells occur in man, and that also here they re present already prenatall

12 huma fetuses (11—33 cm total length) were obtained t legal abortions by hysterotomy. Pieces from duodenum (and th pyloric sphincter) stoma h, and t rminal ileum w re removed from 6 of the fetuses for fluorescence histochemical loc lization of catecholamines nd 5-HT ccording to Falck and Hillarp (see Falck and Owman 1965. Some of the fresh specimen were incubated for 10 min at 37 C in Tyrode sol ion alone in the presence of l g ml l-DOPA. DOPA decarboxylase activity was measured radiometrically Håkanson 1966) in he stomach wall from the rema ning 6 fetuses

In all fetuses a moderate to large number of 5-HT containing enterochromaff cells, emitting a yellow light, were found mainl basall in the mucosa of leum (Fig 1a) and duoden m (Fig 1b). Only scattered cells w th a similar distribution c curred in the stomach wall (Fig 1). The fluorophore which had a coarsel granular appearance was characteristicall concentrated to th basal end of the developing enterochromaffin cell the fluorescent material being ctually absent in the apical neck portion (Fig 1 and b. The ells had the same ppearance whether incubated in T rode s sol tion or not

The yellow fluorescence of the enterochromaff cells seemed to remain nchanged also in the presence of l-DOPA in the incubation med m However green granul r fluorescence had dev loped in the cytopl sm of nother system of epithelial ells, having the same morphology as the 5-HT cells nd loc ted strictly basall in the mucosa (Fig 1b nd). Their number was roughly equal in all three regions, nd lower than that of the ellow fluorescent nterochromaff n cell

There is strong reason to believe (Håkanson t al. 1967a) that the green fluorescence that had developed in the enterochromaffin-lik cells reflects the presence of the decarboxylated product dopamine rather than of DOPA tself Accordingly DOPA decarboxylase could be demonstrated in the stomach wall at all st ges. The

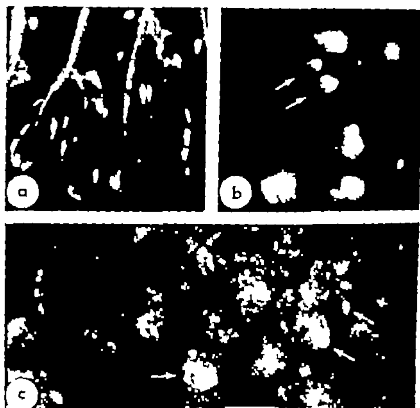


Fig 1 Fluorescence photomicrographs. fetus 28 cm. ( ) Terminal ileum) low-fluorescent enteroch omaffin cells located basally in the mucosa. The fluorophore is restricted to the basal end of the cells Tyrode 180X (b) Duodenum mucosal epithelium with) llow- and greenfluorescent (arrow) ells of the enterochromaffin type the pical "neck portions to the left are virtually not fluorescent Tyrode with l-DOPA 450X ( ) Stomach: Scattered cells of the enterochromaffin type in the mucosa emitting a yellow or green (rrow) fluorescence In most cells the fluorescen material is present in the basal end of the ells. Tyrode with l-DOPA 160X

activ ty per unit weight was found to be approximately one tenth that of the adult rat stomach (Håkanson and Owman 1966)

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